FORM PTO-1390 (REV 11-98)	U.S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 1430-263
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C F R 1 5) 09/763024 Unknown
INTERNATIONAL APPLICATION NO. **PCT/GB99/02738**	INTERNATIONAL FILING DATE **18 August 1999**	PRIORITY DATE CLAIMED **19 August 1998**

TITLE OF INVENTION

GRIP, HUMAN ADAPTER PROTEIN RELATED TO THE GRB2 FAMILY MEMBER

APPLICANT(S) FOR DO/EO/US

ELLIS

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☒ has been transmitted by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   - d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information. **PTO-1449 and copy of International Search Report**

☐ This application is entitled to "Small entity" status. ☐ "Small entity" statement attached.

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
Unknown 09/763024	PCT/GB99/02738	1430-263

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):	
-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2) paid to USPTO $710.00	
-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00	
-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	

ENTER APPROPRIATE BASIC FEE AMOUNT =						$	860.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).						$	130.00
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE			
Total Claims	8	-20 =	0	X	$18.00	$	0.00
Independent Claims	3	-3 =	0	X	$80.00		0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					$270.00	$	0.00
TOTAL OF ABOVE CALCULATIONS =						$	**990.00**
Reduction by ½ for filing by small entity, if applicable. Small entity status must also be asserted. (Note 37 C.F.R. 1.9, 1.27, 1.28).							0.00
SUBTOTAL =						$	**990.00**
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +							0.00
TOTAL NATIONAL FEE =						$	**990.00**
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +						$	0.00
Fee for Petition to Revive Unintentionally Abandoned Application ($1240.00 – Small Entity = $620.00)						$	0.00
TOTAL FEES ENCLOSED =						$	**990.00**
						Amount to be: refunded	$
						Charged	$

a. ☒ A check in the amount of $990.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
d. ☐ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Mary J. Wilson
NAME

32,955 — REGISTRATION NUMBER
February 16, 2001 — Date

JH

PCT09

RAW SEQUENCE LISTING DATE: 08/30/2001
PATENT APPLICATION: US/09/763,024 TIME: 11:34:35

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     10 <140> CURRENT APPLICATION NUMBER: US 09/763,024
C--> 11 <141> CURRENT FILING DATE: 2001-03-20
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     14 <151> PRIOR FILING DATE: 1998-08-19
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     40                  20                  25                  30
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153 225                 230                 235                 240
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163 gtg cgg tgg gcc cgg gcg ctg tat gac ttt gag gcc ctg gag gat gac   864
164 Val Arg Trp Ala Arg Ala Leu Tyr Asp Phe Glu Ala Leu Glu Asp Asp
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169     290                 295                 300
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196     50                  55                  60
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PATENT APPLICATION:  US/09/763,024          TIME: 11:34:35

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217                 165                 170                 175
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220             180                 185                 190
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223         195                 200                 205
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226     210                 215                 220
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229 225                 230                 235                 240
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232                 245                 250                 255
234 Met His Arg Arg His Thr Asp Pro Val Gln Leu Gln Ala Ala Gly Arg
235             260                 265                 270
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238         275                 280                 285
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241     290                 295                 300
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261 ttcactgccc aagccggtgc cacaatgccc atcatttatg tcaaggctgc ctcctcggcg 300
262 ttcctggtgg aaatggtggt gctgcagata tcgctgctgt gggggctgct gcagctgctg 360
263 gggcgctggg gcatattgcg gaggctgtgg ctggtgctgg tgctgctgca ggggaagggt 420
264 cggggggtga tccgacagct tccggttcat cgaaggtcgg atttcttctc ccacagcccc 480
265 actgaggtgt gggcctccct gggacctccg gtccaggctg ttgccccggt gaccctggtc 540
266 ttctcgggtt ctgtctctaa ggaagatctg cttctgtctg gagatggaat ttgtcctgta 600
267 gtagtctacc agcttattta gggaaggaaa cttctcagtc cacagaaagt aattaccctt 660
268 gttgtctcgc atgaccttga agtgttgaac gtcatcctca tgcctgacag agatggagaa 720
269 gtcccctggg gagctctggc tggcccggat gatgaagaag ccaacctcct tgcccatgag 780
270 taagttctct gcctggtgtc gagagaggcc ttcgtgaaac catttgggaa actggatgtc 840
271 tatgaaattc ttgggcacat atccttcctg gctcccaagc tccgccttaa accactcctc 900
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295 Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp
296  15                 20                  25                  30
298 aag tct aga gga tcc aag ctt atg gaa gct gtt gcc aag ttt gat ttc   146
299 Lys Ser Arg Gly Ser Lys Leu Met Glu Ala Val Ala Lys Phe Asp Phe
300                  35                  40                  45
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303 Thr Ala Ser Gly Glu Asp Glu Leu Ser Phe His Thr Gly Asp Val Leu
304              50                  55                  60
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328          35                  40                  45
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VERIFICATION SUMMARY DATE: 08/30/2001
PATENT APPLICATION: **US/09/763,024** TIME: 11:34:37

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L:11 M:271 C: Current Filing Date differs, Replaced Current Filing Date

GRIP, HUMAN ADAPTER PROTEIN RELATED TO THE GRB2 FAMILY MEMBER

Background of the Invention

The stimulation of T lymphocytes by antigen-presenting cells is believed to require activation of two groups of intracellular signalling pathways (Weiss and Imboden, 1987; Allison, 1994). One signal is derived from the ligation of the T cell receptor complex by antigen in association with MHC molecules, resulting in the activation of a number of intracellular protein tyrosine kinases (reviewed in Peri and Veillette, 1994), principally p56lck and ZAP70. These events can lead to the cell entering an activated or a non-responsive state (Quill and Schwartz, 1997; Mueller et al, 1989a; Mueller et al, 1989b; DeSilva et al, 1991; Harding et al, 1992), depending on the presence or absence of a second, or costimulatory, signal resulting from the ligation of various accessory molecules on the T cell surface.

Although a number of cell surface molecules have been shown to possess costimulatory activity, notably CD2, VLA4 and LFA-1 (van Seventer et al, 1991; June et al, 1994a); the best characterised and most potent stimulus arises from the interaction of CD28 on the T cell surface with its counter-receptors CD80 (B7-1; Freeman et al, 1989) and CD86 (B7-2; Freeman et al, 1993a,b; Azuma et al, 1993; Caux et al, 1994) on APC (Harding et al, 1992; June et al, 1994; Freeman et al, 1993a,b; Galvin et al, 1992; Judge et al, 1995). Indeed, exposing peripheral blood CD4-positive T cells to a TCR stimulus such as immobilised anti-CD3 together with CD80 or CD86 is sufficient to drive proliferation (Freeman et al, 1993a,b; Linsley et al, 1991a). Several anti-CD28 monoclonal antibodies also possess stimulatory activity (reviewed in June et al, 1990; June et al, 1994b).

In addition to CD28, many T cells populations also express a second ligand for CD80 and CD86: CTLA4. This molecule shares significant primary structure homology with CD28 (Linsley et al, 1991b). CTLA4 has been shown to bind CD80 and CD86 with a higher affinity than does CD28, a difference of at least 20-fold (Linsley et al, 1991a,b; Freeman et al, 1991; Peach et al, 1994). Activation of CTLA4 appears to produce an inhibition of T cell activation, opposing the stimulatory signal from CD28 (Walunas et al, 1996).

Thus, the operation of the CD28 and CTLA4 pathways is interlinked, and the final outcome of a T cell activation attempt may depend on the relative balance and interplay between the CD28 and CTLA4 signals. To reflect this interlinking, the term 'B7 costimulatory system' is used herein to refer to the integrated system encompassing both CD28 and CTLA4 signals.

The therapeutic potential of agents that manipulate the B7 costimulatory system has been well recognised, and their use has been demonstrated in auto-immune disease, cancer and other conditions of immune dysfunction.

Foremost amongst these agents is the soluble receptor CTLA4-Ig. This consists of the extracellular domain of CTLA4 genetically fused to immunoglobulin constant region, as disclosed in US 5434131 and WO 93/00431. This molecule binds with high affinity to CD80 and CD86, and prevents their association with CD28 and CTLA4 on T cells. It therefore acts as a competitive antagonist of the B7 costimulatory system. Its efficacy has been demonstrated in a wide variety of systems, both *in vitro* and *in vivo* (Linsley et al, 1992; Corry et al, 1994; Wallace et al, 1994; Finck et al, 1994; Ronchese et al, 1994; Perrin et al, 1995), where it has marked beneficial effects in many manifestations of autoimmune disease.

Antibodies which bind to CD80 and/or CD86 and block interactions with CD28 and/or CTLA4 are also well known. These too have been shown to have

beneficial therapeutic utility in models of autoimmune diseases (see for example Keane-Myers et al, 1998; Katayama et al, 1997).

Antibodies which bind to and block the CD80/CD86-binding activity of CTLA4 have also been described. These modulate the B7 costimulatory system by preventing the negative CTLA4 signal from antagonising the stimulatory signal produced when CD28 binds CD80 or CD86. The net result of this modulation is an enhancement of T cell responsiveness. Their utility in the treatment of cancer has also been shown (see for example, Leach et al, 1996).

Antibodies which bind to either CD28 or CTLA4, and provide an activating signal in the absence of ligand are also known. Such antibodies which activate CD28 generally have a net stimulatory effect (see for example Ledbetter et al, 1990), which may result in cellular proliferation and the production of cytokines. Antibodies which activate CTLA4 generally have a net inhibitory effect, and may induce inactivity, anergy or apoptosis (see for example Walunas et al, 1996).

However, all of these approaches to the modulation of the B7 costimulatory system rely on large protein molecules which have many disadvantages as therapeutic agents. Generally they must be administered by injection or infusion as they are poorly orally bioavailable. Furthermore, they may be recognised by the immune system as foreign protein and therefore made the target of an undesired immune response. This may neutralise the biological activity of these polypeptides, or may have deleterious consequences for the host, such as the development of allergy or anaphylactic shock.

As a consequence, a preferred approach to the manipulation of the B7 costimulatory system is through the development of non-polypeptide agents which modulate the activity of the CD28 or CTLA4 pathways. Preferably such agents are easily synthesised small molecular weight chemical entities. These

may modulate the binding of CD28 or CTLA4 by CD80 or CD86 in a manner similar to the polypeptide agents described above.

Alternatively, some such agents may permeate into the cell where they may act to modulate the expression or function of proteins involved in the pathways by which CD28 and CTLA4 affect cellular function. Previous investigations have identified a number of signalling molecules as forming part of the CD28 or CTLA4 signalling pathways, and these could be considered as targets for the activity of agents designed to modulate the B7 costimulatory system. For example, the p85 subunit of PI3-kinase and Grb2 have been shown to associate with CD28 following activation (reviewed in Ward, 1996). CTLA4 has been shown to associate with p85, SHP2, and AP50 (Schneider et al, 1995; Marangere et al, 1996; Zhang and Allison, 1997)

However, these molecules are present in cell types other than T cells and are also components of signalling pathways outside the B7 costimulatory system. Therefore, putative therapeutic approaches that manipulate such signalling molecules have substantial potential for unwanted and undesirable side effects by modulating other pathways. As a more advantageous strategy, it would be better to manipulate the expression or function of a protein which is expressed principally in T cells and which interacts specifically with CD28 or CTLA4.

Summary of the Invention

Accordingly. in one aspect of the present invention, the nucleotide and deduced protein sequence of a protein with exactly these desirable properties is provided. This protein is herein termed GRIP.

In another embodiment of the invention, vectors enabling the expression of GRIP in both eukaryotic and prokaryotic cells are also provided.

In another aspect of the invention, antibodies are provided that specifically recognise GRIP. These antibodies are useful in the detection of GRIP, and hence in the identification of diseases or abnormal cellular states in which modulation of GRIP function or expression may be beneficial.

To facilitate the identification of therapeutic agents which modulate the function of the B7 costimulatory system, other embodiments of the invention provide simple biochemical and immunological assays for the detection of the interaction between GRIP and CD28 and other proteins. It will be apparent to those skilled in the art that these assays may be employed to identify molecular entities capable of disrupting this interaction.

In another aspect of the invention, a PCR-based assay is provided that enables the specific detection of mRNA encoding GRIP from total RNA derived from cells that express GRIP. It will be apparent to those skilled in the art that this assay may be used to identify molecular entities capable of modulating the expression of GRIP mRNA and hence GRIP protein and therefore affecting the operation of the B7 costimulatory system.

Detailed description of the invention

Throughout the following examples of the invention, use is made of various widely known and practised techniques in molecular and cellular biology. Practical details of these may be found in a number of textbooks including Sambrook et al, 1989. Unless otherwise stated, PCR reactions were performed using AmpliTaq enzyme (Roche) in reaction mixes containing buffer and nucleoside triphosphates at the recommended concentrations, and primers at a concentration of 1 □M each. Thermal cycling was performed according to the following general scheme: 5 minutes at 95°C followed by a number of cycles

usually between 10 and 50, each made up of 1 minute at 95°C, 1 minute at an appropriate annealing temperature, most often 50°C, and 1-2 minutes at 72°C. Optionally a further 5 minute incubation at 72°C may be added. The precise times, temperatures and number of cycles may be altered as widely known by those skilled in the art to optimise the reaction yield for the particular thermal cycler, reaction tubes and other practical variables that may apply to any given laboratory. Numbered sequence positions that refer to amino acid residues in human CD28 are according to the scheme adopted by Barclay et al (1997), which is based on the amino acid sequence of full length mature human CD28. Amino acid sequences or designations may be given in either the one letter code, or the three letter code.

Example 1: Cloning of plasmids

A number of modifications to previously published plasmids were undertaken to facilitate cloning. The polylinker of the plasmid pYTH9 (Fuller et al, 1998) was modified by standard molecular biology techniques to insert an in-frame BssHII site. The sequence of the modified polylinker is shown in Figure 1: this plasmid is termed pYTH9/BssHII. A similar procedure was undertaken using the plasmid pAS1CYH2 to create pAS1CYH2/BssHII (Figure 2). A similar procedure was also performed to alter the reading frame of the BamHI site in plasmid pACT2 to create pACT2/BamHI (Figure 3). pAS1CYH2 and pACT2 are standard yeast two-hybrid vectors (Clontech MATCHMAKER kit; Clontech, Palo Alto, CA; Harper et al, 1993)

Four overlapping oligonucleotides (CC205, CC206, CC207 and CC208) which together encode the entire cytoplasmic domain of human CD28 (Figure 4) were mixed in the molar ratios 10:1:1:10 respectively and then subjected to a PCR reaction to generate the full length cytoplasmic domain of human CD28 tagged

with BssHII and NotI restriction sites in the correct reading frame for preparing fusion proteins with GAL4BD.

The resulting PCR product was digested with BssHII and ligated into a variant of pUC18 into which a unique BssHII cloning site had previously been inserted by standard molecular biology techniques. A clone of the expected sequence named pxCD28Y was identified by fluorescent dye-terminator sequencing. pxCD28Y was digested with BssHII and NotI to liberate the CD28 insert. This fragment was purified by preparative agarose gel electrophoresis and then inserted into BssHII-NotI-cut pYTH9/BssHII. A correct clone of the resulting plasmid (pY3H8Y) was identified by sequencing as above. This plasmid therefore encodes a fusion protein consisting of the binding domain (BD) of the GAL4 protein fused in frame to the cytoplasmic domain of human CD28. The same strategy was used to transfer a BssHII fragment bearing the CD28 sequence from pxCD28Y into BssHII digested pAS1CYH2 to create plasmid pCD28Y.BD, also encoding a GAL4BD-CD28 fusion protein.

A sequenced clone of human p85α (Genbank M61906) was used as a template in a PCR with primers CE14 and CE15. The resulting product was digested with BamHI and EcoRI, purified by preparative gel electrophoresis and ligated into BamHI-EcoRI cut pACT2/BamHI. A clone of the correct sequence was identified as above, and termed p85SH2C.AD. This construct encodes a fusion protein consisting of the GAL4 activation domain (AD) fused in frame to a portion of human p85α including the C terminal SH2 domain (GAL4AD-p85SH2)

pY3HY8 was linearised by digestion with XbaI, and transfected into Saccharomyces cerevisiae Ylck4.1 as described (Fuller et al, 1998). Single clones growing in media lacking tryptophan were selected and analysed for the presence of the gene encoding the GAL4BD-CD28 fusion protein. Yeast chromosomal DNA was isolated as described (Fuller et al, 1998) and samples subjected to PCR using primers CC205 and CC208. Several clones showing a

positive signal in the PCR were cultured and cellular protein extracted by boiling in SDS-PAGE sample buffer. Samples were then subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed for the presence of GAL4BD-CD28 fusion protein using a monoclonal antibody against GAL4BD (Santa Cruz cat #SC510). Bound antibody was visualised using a chemiluminescent method as described by the manufacturer (SuperSignal; Pierce). A yeast clone expressing a fusion protein of the expected size was chosen for further work and termed Y8.

Example 2: Identification of GRIP

To identify novel proteins binding to CD28, a variant of the yeast two hybrid technique was employed (Fuller et al, 1998). In the yeast two hybrid, use is made of the fact that the GAL4 transcription factor contains two domains: the activation domain (GAL4AD) and the binding domain (GAL4BD). Formation of a close physical complex between these two domains reconstitutes a transactivating activity which can be employed to activate expression of suitably prepared reporter genes. These two domains can be expressed separately as fusion proteins with heterologous proteins, in such a fashion that if the heterologous fusion partners form a complex with each other, the two GAL4 domains are brought into apposition and become active. If the fusion partners do not interact, there is no such activity. Hence, this system forms a powerful tool for the determination of whether two arbitrary proteins interact. This principle is well known in the field, and there are a number of variants and applications which have been described (see for example Fuller et al, 1998).

Yeast strain Y8 contains the GAL4BD-CD28 fusion protein and also catalytically active murine Lck derived from its parent Ylck4.1 (Fuller et al, 1998). The Lck gene is expressed under a regulated promoter such that it is expressed in the absence of methionine in the culture medium and repressed by the presence of

2mM methionine (Fuller et al, 1998). Since the CD28 cytoplasmic domain contains a residue (corresponding to tyrosine 173 in mature human CD28) which is a substrate for Lck (see for example King et al, 1997), it was predicted that in the presence of the tyrosine kinase in the yeast, the fusion protein would become phosphorylated at this site. Furthermore, it is also known that CD28 bearing phosphotyrosine at this site is bound by the p85 subunit of PI3 kinase, via its SH2 domains (see for example Raab et al, 1995). By analogy with previous experience in this system, we predicted that association of tyrosine phosphorylated GAL4BD-CD28 fusion protein with GAL4AD-p85SH2 would recreate GAL4 transactivating activity, causing expression of the LacZ and HIS3 reporter genes present in this yeast strain (Fuller et al, 1998). No transactivation would be predicted in the absence of Lck, as SH2 domains require phosphotyrosine for their binding site.

Yeast clone Y8 expressing the GAL4BD-CD28 fusion protein was transfected with either plasmid p85SH2C.AD or plasmid pACT2 and transformants selected on media deficient for tryptophan and leucine (Met+ media) or media deficient for tryptophan, leucine and methionine (Met- media). Samples of growing yeast were assayed for LacZ activity as described (Fuller et al, 1998). Only Y8 yeast transfected with p85SH2C.AD and growing on Met- media showed LacZ activity, indicating that GAL4BD-CD28 interacts with GAL4AD-p85SH2 but not GAL4AD. Furthermore, this interaction was contingent on the coexpression of catalytically active Lck.

These results are exactly as predicted, and indicate that this yeast system accurately recreates the previously observed regulated interaction between CD28 and p85. Based on this validation, we then used the Y8 yeast to screen a pACT2 GAL4AD fusion protein expression library for potential novel binding partners for phosphorylated CD28. This was done essentially as described (Fuller et al, 1998), using the same library. A number of clones were obtained that expressed LacZ activity only when the Lck gene was expressed. Plasmid

DNA encoding the GAL4AD fusion was recovered from each clone and sequenced by fluorescent dye-terminator sequencing. One clone, termed Y8.41, yielded a plasmid herein termed pY3HY8.41 which contained a sequence previously unknown, and was selected for further analysis.

Sequence analysis of pY3HY8.41 revealed an open reading frame in addition to the vector-derived sequence. The deduced amino acid sequence contained motifs characteristic of SH2 and SH3 domains. The 5' end of the insert did not encode a methionine in the correct context to be a start residue, and also contained sequence homologous to a C terminal fragment of an SH3 domain. We therefore postulated that the pY3HY8.41 clone was incomplete, and that further 5' sequence was required to obtain the full coding sequence.

The nucleic acid sequence was used to screen the Genbank database, and two highly similar partial sequences of previously unknown function identified. These were found to overlap the 5' end of the pY3HY8.41 sequence, allowing the preparation of a longer consensus sequence. Upon analysis, this sequence was found to contain both a methionine in a context close to that required for optimal translation initiation (Kozak, 1984) and also the missing portion of the predicted SH3 domain. Based on this consensus sequence, PCR primers CE65 and CE66 were designed to amplify the full length coding sequence for the pY3HY8.41 insert. Total RNA was prepared from 10^6 actively growing Jurkat T cells using the Promega SV Total RNA Isolation kit according to the manufacturer's instructions. Aliquots of the RNA were then subjected to RT-PCR using primers CE65 and CE66 and the Promega Access kit according to the manufacturer's instructions. The resulting PCR product was digested with BssHII and NotI, purified by preparative gel electrophoresis and ligated into appropriately prepared pYTH9/BssHII. Clones containing inserts of the expected size were analysed by fluorescent dye-terminator sequencing, and a complete DNA sequence obtained (Figure 5).

This sequence matches the sequence obtained from pY3HY8.41 and also the consensus sequence prepared using the combination of pY3HY8.41 and the partial sequences from Genbank. Analysis of the deduced protein sequence reveals the presence of two putative SH3 domains, one at the N terminal encoded by cDNA residues 13 to 156, and one at the C terminus of the protein encoded by residues 832 to 990. Between these lie a putative SH2 domain (residues 172 to 441) and another domain of unknown function (residues 442 to 831), rich in proline, glutamine and histidine residues. This latter domain we term the 'insert domain'. The schematic format of the full length protein is therefore N-SH3-SH2-Insert-SH3-C. It will be apparent to those skilled in the art that the boundaries of the domains delineated above are provided simply for ease of reference, and that fragments of sequence inside these boundaries may be deleted, or sequence outside these boundaries added, without detracting from the properties of the domain.

A further search of the sequence databases revealed that the most closely-related known proteins are Grb2 (Genbank M96995; Lowenstein et al, 1992) and Grap (Genbank U52518; Feng et al, 1996). Both of these have the schematic structure N-SH3-SH2-SH3-C, ie they lack a domain homologous to the insert domain. We therefore named the novel full length gene GRIP, standing for Grb2-Related with Insert Protein. The plasmid containing the complete insert, we termed pGRIP.BDI. Since the insert domain is unique to GRIP, it is likely to possess interesting and particular properties not present in other proteins.

Our analysis of the GRIP cDNA sequence indicates that it should encode a protein of approximate molecular weight 38 kilodaltons. Those skilled in the art will know that this may be subsequently altered by post-translational modifications such as phosphorylation, myristylation, palmitoylation, glycosylation, proteolytic cleavage for example. There does not appear to be a sequence consistent with signal sequences which direct the export of proteins from the cell. There are also no sequences with the characteristics of

membrane-spanning stretches of polypeptide. GRIP is therefore most likely to be a cytoplasmic protein, like its closest relatives Grb2 and Grap. The SH3 and SH2 motifs are characteristic modules implicated in protein:protein interactions, and suggest that GRIP is one of a class of proteins known as adapter proteins. Such proteins play critical roles in a wide range of signal transduction pathways (reviewed in Birge et al, 1996). The SH3 domain interacts with proteins containing sequences of the general format Pro-Xxx-Xxx-Pro (where Pro is proline and Xxx is any amino acid residue, also sometimes denoted PXXP sequences using the one letter amino acid code) (reviewed in Musacchio et al, 1994). SH2 domains interact with phosphotyrosine containing motifs (reviewed in Schaffhausen, 1995) generated by the activity of protein tyrosine kinases upon substrate proteins. The insert domain may have a similar role in protein:protein interactions, or it may have an enzymic function or a nucleic acid binding function or it may have a role in determining the three-dimensional disposition of other parts of GRIP or it may have some other function.

Since a major function of such adapter proteins is to associate with other proteins and to modulate their function or localisation, it will be apparent that either other chemical entities or mutations in GRIP which modulate the ability of GRIP to bind other proteins may be employed to therapeutic effect to modulate the function of pathways in which GRIP plays a role. These may target individual domains in GRIP, individual binding sites in or for GRIP, or may affect other portions of GRIP, or even the entire protein.

It should be noted that sequences other than that laid out in Figure 5 may also be determined, perhaps revealing deletions, additions or mutations in any given GRIP cDNA clone or mRNA or genomic DNA sample. These changes may or may not affect the deduced amino acid sequence of the individual GRIP clone. Such variant sequences that are substantially the same as GRIP do not depart from the scope of the present invention. Such variants that are substantially the same will generally have amino acid similarities to GRIP which may exceed the

level of 99% or 95% or 90% or 85% or 80% or 70% or 50%. Furthermore, there may also be GRIP sequences in which one or more exons have been removed, replaced or added by alternative splicing. These too are encompassed by the present invention.

Example 3: Localisation of GRIP expression

Since the related protein Grb2 is ubiquitously expressed, we were interested to examine the tissue distribution of GRIP expression. We prepared primers CE71 and CE72 which hybridise to residues 430 to 453 and 802 to 825 respectively in the sequence of Figure 5. These were employed in PCR using as template cDNA prepared from mRNA extracted from a variety of normal adult tissues. cDNA obtained from skin, brain, liver, colon, skeletal muscle, testis and lung was obtained from Invitrogen (Discovery Line; Invitrogen;). cDNA libraries prepared from normal human lymph node and spleen were a kind gift of Dr E Zanders, GlaxoWellcome, UK. In parallel control reactions, we also employed primers CE102 and CE103 specific for a portion of the GAPDH message (a ubiquitously expressed gene the presence of whose cDNA in a library is widely used as a marker for both success of a PCR reaction and of the cDNA preparation).

PCR reactions contained each primer at a final concentration of 1 μM, 1.25 units of AmpliTaq (Roche), 1 μl of cDNA and nucleoside triphosphates and buffers at the concentrations recommended by the enzyme supplier (Roche). The reactions were placed in a thermal cycler (Trio, Biometra) and subjected to 5 minutes at 95°C followed by a number of cycles, each made up of 1 minute at 95°C, followed by 1 minute at 50°C, followed by 1 minute at 72°C. Reactions with GRIP primers used 45 cycles, those with GAPDH primers, 35 cycles.

At the end of the reaction, 10 μl samples of each reaction were analysed by agarose gel electrophoresis. The results are shown in Figure 6. All of the

GAPDH reactions showed strong signals, indicating successful cDNA preparation and ubiquitous expression of GAPDH. In contrast, specific signals were only observed in two of the GRIP reactions: those using spleen and lymph node cDNA as template. These data indicate that GRIP expression is confined to lymphoid tissue.

Since lymphoid tissue contains many cell types (T cells, B cells, monocyte lineage cells for example), we next prepared total RNA as described previously from Jurkat (a T cell line), OZZ and MAW (two B cell lines) and Thp1 (a monocyte lineage cell line). RT-PCR reactions were prepared using the Promega Access RT-PCR kit according to the manufacturer's instructions (Promega, Madison, WI). Each reaction contained 1 μM final concentration of each of two primers, either CE71 and CE72 for GRIP or CE102 and CE103 for GAPDH. Cycling was performed according to the manufacturer's instructions, using 45 cycles for each reaction. Upon completion, 10 μl samples were analysed by agarose gel electrophoresis. The results are shown in Figure 7, and demonstrate that GRIP mRNA is only expressed in the T cell line. We therefore believe GRIP expression to be primarily confined to T cells.

It will be apparent to the person skilled in the art that these or similar primer sequences and PCR or RT-PCR reactions (or other amplification or hybridisation technologies, as well known and widely practised), may be employed to specifically determine the level of GRIP mRNA in the manner of a diagnostic kit. GRIP mRNA overexpression may be associated with conditions of inappropriate immune system activity, such as autoimmune diseases like rheumatoid arthritis, psoriasis, allergic asthma. Similarly, GRIP mRNA under-expression may be associated with conditions of insufficient immune system activity such as cancer, or immunosuppression.

Moreover, although for our convenience we have used particular methods for the detection of GRIP mRNA or cDNA, it will be obvious to those skilled in the art

that suitable probe sequences may be derived from the teaching presented herein to allow detection of GRIP mRNA, cDNA, genomic DNA derived from human or other species by standard methods including Northern blotting or Southern blotting. Also, under appropriate experimental conditions, the same may be used to enable the detection, characterisation or purification of polynucleotide sequences closely related to GRIP by virtue of the possession of sufficient homology to allow selective hybridisation to one or more probe sequences.

Example 4: Regulation of GRIP expression

To determine whether GRIP expression was modulated by external stimuli, we isolated and purified peripheral blood CD4-positive T lymphocytes as previously described (Ellis et al, 1996). These cells were stimulated for varying periods of time with activating antibodies against CD3 (mAb OKT3) and CD28 (mAb 9.3) immobilised on a plastic tissue culture well. After the specified period of time, the cells were immediately lysed and total RNA prepared from the lysates as described above. Two cohorts of cells were not lysed at the end of their period, but instead were incubated with ^{3}H-thymidine as part of a standard thymidine incorporation assay for cell proliferation as a control for successful stimulation. One cohort was not treated with the activating antibodies, the other was exposed to them for 48 hours. The results of this are shown in Figure 8, and clearly show that the activating antibody treatment was successful in activating the cells to proliferate.

Samples of each total RNA preparation were then analysed by RT-PCR for GRIP and GAPDH mRNA levels essentially as described above, except that in the GRIP RT-PCRs, primers CE65 and CE66 were used, and the reactions received 50 cycles. The results are also shown in Figure 8. These data demonstrate that while GRIP mRNA is present in unactivated resting cells, its

level markedly increases upon cell activation, in excess of a small general increase in cell mRNA as evidenced by an increase in the GAPDH signal.

One skilled in the art can readily see how this assay may be adapted and utilised to search for agents which specifically modulate the levels of GRIP mRNA in some desired fashion, perhaps increasing the amount of GRIP mRNA or decreasing the amount of GRIP mRNA. Furthermore, by utilising the RT-PCR for GAPDH mRNA, it is possible to distinguish agents which specifically act on GRIP mRNA levels, rather than more generally modulating mRNA levels within the cell. Such agents may include antisense RNA or DNA, triplex-forming oligonucleotides, ribozymes and similar agents well known to those in the field.

Example 5: Expression of GRIP in eukaryotic cells

To confirm that the full-length GRIP insert did indeed encode a translatable protein, it was transferred to a eukaryotic expression vector. A small amount of plasmid pGRIP.BDI was used as template in a PCR reaction with primers CE65 and CE79 which attach a number of restriction sites. The resulting PCR product was digested with BamHI and EcoRI and ligated into appropriately prepared pcDNA3.1HisC (Invitrogen; Holland). This plasmid provides two short peptide tags (the Xpress tag and the His6 tag) at the N-terminus of the inserted protein which may be used for identification or purification of the protein. The resulting plasmid, pGRIPFL.His, was analysed by fluorescent dye-terminator sequencing, and found to have a small deletion, creating a spurious BamHI site within one of these tags, such that the downstream GRIP insert was thrown out of the reading frame.

To correct this error, a pair of complementary oligonucleotides CE106 and CE107 were designed, annealed together, and then cloned into pGRIPFL.His at the spurious BamHI site. The resulting plasmid, pGRIPFL.Fix was analysed by

sequencing as above, and found to have the predicted structure. The sequence of the 5' end of this construct is shown in Figure 9, illustrating the nature of the junction with the vector-encoded tag sequences.

To prepare this construct in a form suitable for expression in eukaryotic cells, a sample of pGRIPFL.Fix was then used as the template in a PCR using primers CE108 and CE109. These attach a consensus translation initiation sequence upstream of the initial methionine codon, and also attach EcoRI cloning sites to the ends of the insert. The resulting PCR product was digested with EcoRI and cloned into suitably prepared pCI (Promega). pCI contains a promoter and other elements required for expression of inserts in eukaryotic cells. Clones were analysed by restriction digestion, and one showing the predicted pattern of restriction sites taken for further analysis. The structure of this plasmid, pGRIP-X was then confirmed by fluorescent dye-terminator sequencing. A similar construct, pGRIP-H, bearing the HA-tag in place of the Xpress and His6 tags was prepared in a similar fashion, using primer CE110 in place of CE108.

Samples of pGRIP-X, pGRIP-H, empty parental pCI vector or pcDNA3.1HisLacZ (a control plasmid containing an expressible gene for another protein tagged with the Xpress epitope; Invitrogen) were transfected into COS1 cells using lipofectamine according to the manufacturer's protocol (Gibco BRL). After 48 hours the cells were lysed and prepared for SDS-PAGE according to standard protocols. Samples were separated on 8-16% acrylamide gradient gels (Novex), blotted to nitrocellulose and then probed for the presence of the Xpress tag using an anti-Xpress mAb (Invitrogen) according to standard Western blotting protocols. Bound antibody was visualised as described above. The results are shown in Figure 10, and reveal that pGRIP-X specifically directs the expression of a circa 45kDa protein containing the Xpress tag. This is in good agreement with the predicted molecular weight of the GRIP protein once the size of the tags has been accounted for. These data demonstrate that the GRIP insert indeed encodes a fully translatable protein.

Example 6: Expression of GRIP in prokaryotic cells

To prepare a convenient supply of GRIP protein for biochemical and other studies, the GRIP insert was prepared for cloning into the pGEX4T3 vector (Pharmacia). This vector expresses correctly inserted protein as a fusion with glutathione S-transferase, by means of which it may readily be purified by glutathione sepharose affinity chromatography.

pGRIP.BDI was used as template in three PCR reactions, each designed to tag a particular portion of GRIP sequence with BamHI and EcoRI cloning sites in the appropriate reading frame such that when the resulting fragment was cloned into pGEX4T3, a GST-GRIP fusion protein would be produced.

In one reaction, primers CE65 and CE79 were employed to amplify full length GRIP cDNA, here termed GRIPFL. In a second reaction, primers CE69 and CE70 were used to amplify a portion of GRIP cDNA encompassing the residues coding for the putative SH2 domain (residues 151 to 459 of Figure 5), here termed GRIPSH2. In the third reaction, primers CE71 and CE72 were used to amplify a portion of GRIP cDNA encompassing the residues coding for the insert domain (residues 430 to 825 of Figure 5), here termed GRIPINS.

Each of the three reaction products was digested with BamHI and EcoRI, purified by preparative gel electrophoresis, and ligated into suitably prepared pGEX4T3 to create constructs pGRIPFL.GEX, pGRIPSH2.GEX and pGRIPINS.GEX respectively. Single clones of each of these were analysed by fluorescent dye-terminator sequencing and confirmed to have the expected structure. These plasmids therefore encode GST-GRIP fusion proteins, termed GST-GRIPFL, GST-GRIPSH2 and GST-GRIPINS respectively.

These plasmids were transformed into E. coli strain BL21 (Novagen) and bacterial cultures initiated from single transformant colonies. As a control, parental pGEX4T3 was also transformed into BL21 bacteria and used to initiate cultures: the product of this plasmid is here termed GST. A 200 ml mid-log phase culture of each construct was prepared, and expression of the GST fusion proteins induced by the addition of IPTG to a final concentration of 1 mM in the culture medium. After further growth for approximately 3 hours at 30°C, bacteria were recovered by centrifugation. Bacterial pellets were resuspended in 2.5 ml of either PBS/1% Triton X100 or 25 mM Tris pH8.0/1 mM EDTA/1% Triton X100/0.2% NP40/1 mg/ml lysozyme, each supplemented with Complete protease inhibitors used according to the manufacturer's instruction (Boehringer-Mannheim), and then lysed by sonication. Insoluble matter was removed by centrifugation, and the clarified lysates mixed with 0.67 ml glutathione sepharose 4B previously washed according to the manufacturer's instructions (Pharmacia). These reactions were tumbled for 30 minutes at room temperature, the resin pelleted by centrifugation, and the supernatant discarded. The resin in each sample was then washed with 25 ml 2 mM EDTA/PBS.

In some experiments, the resulting resins were then stored in 2 mM EDTA/PBS at 4°C for short periods of time for later use. These we term 'charged resins', and contain GST or GST-GRIP fusion proteins immobilised onto the glutathione sepharose 4B, and are useful for affinity chromatography protocols involving proteins which associate with GRIP. In other experiments, the GST fusion proteins were eluted from the resin by incubation with 10 mM reduced glutathione/PBS elution buffer, separated from the resin by centrifugation, and stored frozen at –20°C as purified protein. Both charged resins and purified protein were prepared for GST-GRIPFL, GST-GRIPSH2, GST-GRIPINS and also GST alone (using the parental pGEX4T3 transfected cells).

Samples of these purified proteins were analysed by SDS-PAGE and staining with Coomassie stain. Each preparation contained a major protein band of the

size expected for each GST fusion protein, and sometimes a number of faint lower molecular weight bands reflecting partial degradation products.

Example 7: Production of monoclonal antibodies against GRIP

Samples of GST-GRIPFL were used to immunise mice according to a published protocol (Kilpatrick et al, 1997). Hybridoma fusions were prepared, cultured and maintained as described (Kilpatrick et al, 1997). Samples of hybridoma supernatant were used as probe antibody in Western blots of SDS-PAGE gels upon which samples of GST-GRIPFL and GST had been separated. Those cultures whose supernatants showed preferential immunoreactivity for GST-GRIPFL as against GST were selected for further analysis. Cultures whose supernatants additionally showed immunoreactivity for the Xpress-tagged GRIP molecule present in pGRIP-X-transfected COS1 cell lysate as described above were further selected.

From a number of these cultures, clonal populations of hybridomas were prepared by limiting dilution cloning, and the culture supernatants of these clones analysed as described above. A number of clones were chosen, whose supernatants showed immunoreactivity on Western blots for GST-GRIP and Xpress-tagged GRIP, but not for GST. These supernatants therefore contain monoclonal antibodies reactive with GRIP.

One of these monoclonal antibodies, 1-13.4, was employed in another Western blot. In this, cell lysates were prepared according to standard protocols from Jurkat T cells. Samples of these lysates were separated by SDS-PAGE, along with samples of GST-GRIPFL, transferred to nitrocellulose and then probed using the anti-GRIP monoclonal antibody 1-13.4. Bound antibody was visualised as described above. The results are shown in Figure 11. The antibody specifically recognises the GST-GRIPFL protein, and also a single band in the

Jurkat lysate at the molecular weight predicted from the GRIP cDNA sequence. No reactivity was observed at molecular weights characteristic of Grb2 or Grap, even though both are expressed in Jurkat cells.

These data illustrate that monoclonal antibodies prepared using GST-GRIPFL may be used for the detection of natively expressed GRIP and recombinant GRIP, and further, that such antibodies may be specific for GRIP, showing no cross-reactivity for the related proteins Grb2 and Grap.

It will be apparent to those skilled in the art that aspects of the present invention such as GST-GRIPFL may be also used in the production, characterisation and purification of other agents which show specific protein binding activity for GRIP or fragments or variants thereof. These may include polyclonal antisera, antibody fragments such as Fab, Fab2, single chain Fv, humanised antibodies, chimaeric antibodies, bispecific antibodies, other antibody derivatives, binding agents derived from polynucleotides such as aptamers, and other similar agents well known to those skilled in the area.

Example 8: GST-GRIP binds to a CD28 phosphopeptide

To provide a convenient assay for the function of GRIP, an assay was prepared in which the binding of GST-GRIPFL, GST-GRIPSH2, GST-GRIPINS and GST to peptides derived from a portion of the CD28 cytoplasmic domain could be assessed. Two peptides corresponding to the CD28 sequence around tyrosine 173 were chemically synthesised. These had the sequences [biotin]-KLLHSDYMNMTPR ('control peptide') and [biotin]-KLLHSDpYMNMT ('phosphorylated peptide') where [biotin]-K indicates a lysyl residue bearing a biotin moiety and pY indicates a phosphotyrosine residue. The substantive difference between these sequences lies in the presence or absence of a phosphate group attached to the tyrosine residue.

Nunc Maxisorp microtitre plates were coated with 2 □g/ml of streptavidin (STAR1B; Serotec; Kidlington; UK) in PBS, 100 □l per well, and stored overnight at 4°C. After washing with TBS/0.1% Tween 20, unoccupied protein binding sites on the plate were blocked by incubation with 200 □l per well of a 3% w/v solution of BSA in PBS overnight at 4°C. After further washing as above, wells were exposed to an approximate 5 □M solution of either peptide in PBS, 100 □l per well for approximately 1 hour at room temperature. Plates were washed once again, and then exposed to approximately equal concentration solutions of GST-GRIPFL, GST-GRIPSH2, GST-GRIPINS or GST, 100 □l per well for approximately 1 hour at room temperature. Plates were washed once again, and then exposed to a 1/1000 v/v solution of goat anti-GST antiserum (Pharmacia) in PBS, 100 □l per well for approximately 45 minutes at room temperature. Plates were washed once again, and then exposed to a horseradish peroxidase-conjugated antiserum directed against goat Ig (A5420; Sigma Chemical Co) at a concentration of 1/5000 v/v in PBS, 100 □l per well for approximately 45 minutes at room temperature. Plates were washed for a final time, and the bound peroxidase activity quantitated by use of a chromogenic substrate (Fast OPD; Sigma Chemical Co) according to the manufacturer's instructions. After the chromogenic reaction had proceeded to an appropriate extent, it was terminated by the addition of 3M sulphuric acid, 25 □l per well, and the amount of reaction product quantitated by determining the absorbance at 490 nm.

The results are shown graphically in Figure 12. These data show that none of the GST fusion proteins associate with the unphosphorylated CD28 peptide. GST-GRIPFL and GST-GRIPSH2, but not GST-GRIPINS or GST bind the phosphorylated CD28 peptide, showing a specific interaction between phosphorylated CD28 sequences and GRIP.

These data demonstrate that the GRIP SH2 domain is sufficient to mediate interaction with the region of CD28 sequence centred around phosphorylated

tyrosine 173. Furthermore, this interaction is absolutely contingent on phosphorylation of the tyrosine, exactly as observed in the earlier yeast work.

These data also demonstrate that portions of the GRIP sequence may be separated from the whole protein, expressed in a heterologous prokaryotic system, and yet retain biochemical function. From this it is clear that either full length GRIP or various portions or fragments of GRIP, preferably the SH3, SH2 and insert domains, may be used, either alone or in combination with each other or with other proteins (either in their entirety or fragments thereof) to exploit some property of GRIP. For some applications, fragments may be superior to the full-length GRIP protein. For example, a polypeptide including the GRIP SH2 domain, may be used to modulate the interaction of phosphorylated CD28 or other phosphoproteins with natively expressed GRIP. In another example, sufficient fragment of GRIP to bind a given partner protein might be combined with a detectable marker in order to facilitate detection of GRIP-binding partner proteins, or with an enzyme such as a protease in order to target the enzyme activity to the GRIP-binding partner protein. Such combinations and fusions may be accomplished by genetic engineering, in which chimaeric genes encoding the desired polypeptide are constructed, or by crosslinking preformed proteins, or by other similar approaches well known to those skilled in the art. Furthermore, it is also contemplated that deletions, additions or mutations may be made to the GRIP fragments in order to optimise their properties for the desired purpose.

It will be readily apparent to one skilled in the art that such simple biochemically defined assays may be easily adapted and utilised to screen for agents that specifically modulate the interaction between full length GRIP or fragments of GRIP and CD28 or other proteins or peptides derived therefrom.

Furthermore, these data also suggest that agents like the CD28 phosphorylated peptide will act as modulators of GRIP SH2 domain function. In this particular instance, the CD28 phosphorylated peptide will act as an antagonist of GRIP

binding to full length CD28, by means of its affinity for the GRIP SH2 domain. Modifications may be made to this or similar peptides to add or modify desirable properties such as cell permeability, oral bioavailability, stability, affinity, specificity; or to eliminate or ameliorate undesirable properties; without departing from the scope of the invention.

Example 9: GRIP binds specifically to CD28

To elucidate further the specificity of the interaction between GRIP and CD28, experiments were conducted in the yeast two hybrid system to compare the interactions between GRIP and CD28 or CD3□□ Like CD28, CD3□ is another T cell molecule which is phosphorylated by Lck and also forms the site of attachment for SH2 containing signalling proteins.

The BamHI-EcoRI fragment of full length GRIP used in the construction of pGRIPFL.GEX was also ligated into suitably prepared pACT2/BamHI to generate a construct termed pGRIPFL.AD which encodes a GAL4AD-GRIPFL fusion protein. Individual clones of this construct were sequenced to confirm their structure.

pGRIPFL.AD plasmid DNA was cotransfected into Ylck4.1 yeast (Fuller et al, 1998) with pCD28Y.BD plasmid DNA or pAS2/TCR□ plasmid DNA (Fuller et al, 1998). This latter plasmid, a kind gift of Dr MJ Sims, GlaxoWellcome UK, contains an in-frame fusion of GAL4BD to the cytoplasmic domain of human CD3□. Transformants were grown on either Met+ or Met- media, to either repress or induce expression of the Lck protein tyrosine kinase. Resulting colonies were analysed for LacZ activity as described (Fuller et al, 1998). The results are shown in the table below, scored according to the amount of LacZ activity seen. – indicates no LacZ activity, + indicates trace amounts of LacZ

activity, ++, +++ and ++++ indicate progressively stronger amounts of LacZ activity.

GAL4BD plasmid	Lck expression	
	Induced	Repressed
pCD28Y.BD	++++	-
pAS2/TCR□	-	-

These data indicate that full length GRIP interacts strongly with CD28 cytoplasmic domain only in the presence of Lck which is capable of phosphorylating the residue corresponding to tyrosine 173. However, GRIP does not associate with the cytoplasmic domain of CD3□, regardless of the presence or absence of Lck, which is capable of phosphorylating a number of tyrosine residues in CD3□. In a parallel control preparation, Ylck4.1 yeast containing both the pAS2/TCR□ plasmid and also the pACT2/ZAP70SH2 plasmid (Fuller et al, 1998) were analysed: ++++ LacZ activity was obtained upon induction of Lck, - LacZ activity when the Lck was repressed.

These data demonstrate that GRIP interacts specifically with the CD28 cytoplasmic domain, but not generally with other tyrosine-bearing signalling domains which are also capable of being phosphorylated by Lck. These data suggest that agents which modulate the function of GRIP are likely to have specific effects on the CD28 signalling machinery. This specificity greatly increases the therapeutic utility of the present invention, as it provides a route for obtaining a desired effect on the B7 costimulatory system, without adversely affecting other T cell control systems.

Although we have only examined T cells as an example of a cell where both CD28 and GRIP are expressed, there are preliminary reports of a small number of other cell types in which CD28 mRNA or protein may be found, including mast

cells and plasma cells. Such cell types may also represent targets for interventions aimed at modulating a CD28-dependent pathway by manipulating the function of GRIP.

Example 10: Mutational analysis of the GRIP-CD28 interaction

To further delineate the molecular nature of the interaction between GRIP and CD28, a further series of vectors encoding GAL4BD-CD28 fusion proteins were prepared in which one or more residues of the CD28 sequence were mutated in such a fashion as to alter the polypeptide sequence.

Plasmid pCD28F.BD was constructed as described for pCD28Y.BD, except that primer CC209 was used in place of primer CC206, and that the BssHII fragment bearing the CD28 sequence was ligated directly into suitably prepared pAS1CYH2/BssHII. Clones of the correct structure were identified by restriction digestion and sequencing. This plasmid encodes a GAL4BD-CD28 fusion protein in which the tyrosine residue corresponding to CD28 residue 173 is replaced by a phenylalanine residue. This fusion protein is therefore not a substrate for tyrosine phosphorylation at this site.

Plasmid pCD28V.BD was constructed as described for pCD28F.BD, except that primer CE42 was used in place of CC205 and primer CE44 in place of CC206. This plasmid encodes a GAL4BD-CD28 fusion protein in which the methionine residue corresponding to CD28 residue 174 is replaced by a valine residue.

Plasmid pCD28K.BD was constructed as described for pCD28F.BD, except that primer CE42 was used in place of CC205 and primer CE45 in place of CC206. This plasmid encodes a GAL4BD-CD28 fusion protein in which the asparagine residue corresponding to CD28 residue 175 is replaced by a lysine residue.

Plasmid pCD28VK.BD was constructed as described for pCD28F.BD, except that primer CE42 was used in place of CC205 and primer CE46 in place of CC206. This plasmid encodes a GAL4BD-CD28 fusion protein in which the which the methionine residue corresponding to CD28 residue 174 is replaced by a valine residue and the asparagine residue corresponding to CD28 residue 175 is replaced by a lysine residue.

The various GAL4BD-CD28 fusion protein plasmids were then cotransfected into Ylck4.1 yeast along with either pGRIPFL.AD (encoding a GAL4AD-GRIP fusion protein) or p85SH2C.AD (encoding a GAL4AD-p85 SH2 domain fusion protein). Transformants were grown on either Met+ or Met- media, to either repress or induce expression of the Lck protein tyrosine kinase. Resulting colonies were analysed for LacZ activity as described (Fuller et al, 1998). The results are shown in the table below, scored according to the amount of LacZ activity seen. – indicates no LacZ activity, + indicates trace amounts of LacZ activity, ++, +++ and ++++ indicate progressively stronger amounts of LacZ activity.

GAL4AD plasmid	GAL4BD plasmid	Lck expression	
		Induced	Repressed
pGRIPFL.AD	pCD28Y.BD	+++	-
"	pCD28F.BD	-	-
"	pCD28V.BD	++	-
"	pCD28K.BD	-	-
"	pCD28VK.BD	-	-
p85SH2C.AD	pCD28Y.BD	++++	-
"	pCD28F.BD	-	-
"	pCD28V.BD	+++	-
"	pCD28K.BD	+++	-
"	pCD28VK.BD	+++	-

These data demonstrate that the GRIP-CD28 interaction is critically dependent upon both the presence of a tyrosine residue at position 173 and the presence of active Lck. In combination with the result of the experiment described earlier using phosphorylated and unphosphorylated CD28 peptides, these data indicate that GRIP interaction with CD28 is most likely dependent upon the interaction of the GRIP SH2 domain with the sequence surrounding phosphorylated tyrosine 173. Although the CD28 cytoplasmic domain has three other tyrosine residues, each of which potentially may be phosphorylated by Lck, and subsequently potentially bound by GRIP SH2 domain, this experiment shows that only tyrosine 173 is so used. Although GRIP has two SH3 domains potentially capable of interacting with PXXP motifs, of which there are two in the CD28 cytoplasmic domain, any such interaction is insufficient to drive association of GRIP and CD28 in this system.

Furthermore these results also demonstrate that alterations of the amino acid sequence in the near vicinity of the phosphorylated tyrosine residue can dramatically alter the efficiency of association with GRIP. In particular, alterations in the amino acid sequence at position +2 relative to the phosphotyrosine have a particularly strong effect (phosphorylated tyrosine is position 0 in this numbering system). The control experiments using GAL4AD-p85SH2 fusion proteins demonstrate that these alterations do not prevent either phosphorylation of tyrosine 173 or binding by SH2 domains per se: they are having a specific effect upon the association of GAL4AD-GRIP with GAL4BD-CD28.

These findings provide a molecular basis for the specificity of GRIP binding to CD28 demonstrated previously: GRIP association with partner proteins is dependent upon the precise sequence of the partner protein. These data also demonstrate that small changes in this sequence and hence the tertiary structure of the GRIP binding site can be sufficient to completely abrogate association with

GRIP. It will be obvious therefore that agents which are capable of inducing such distortions in the three-dimensional structure of the binding site, perhaps by themselves binding to this area, will serve to modulate the association of GRIP with its partner proteins. Indeed, a polypeptide containing a portion of GRIP sufficient to bind its partner proteins would itself serve as such an agent, modulating the interaction of such partner proteins with native GRIP. Such agents may be used to modulate the function of cells in which GRIP or proteins which interact with GRIP are expressed.

Likewise, it is also obvious that mutations within GRIP which serve to distort the tertiary structure of the parts of the GRIP molecule may also modulate or abrogate the association between GRIP and its partner proteins. Furthermore, it also follows that agents which act to so distort the tertiary structure of GRIP will also be capable of such effects. Such agents may be used to modulate the function of cells in which GRIP is expressed.

Furthermore, such simple assays as that presently described may be adapted in fashions obvious to those skilled in the art so as to serve as a screen for the identification of the modulatory agents described above.

Example 11: Cooperativity analysis of the GRIP-CD28 interaction

Although we have demonstrated that any putative interactions between the SH3 domains of GRIP and the PXXP motifs within CD28 are insufficient to mediate association in the yeast system in the absence of a phosphotyrosine-SH2 interaction, this does not exclude the possibility that such an SH3-based interaction may occur, and may contribute to the total binding affinity between GRIP and CD28.

To assess this possibility, we compared the relative efficiencies of binding of GRIP to either wild-type CD28 or a CD28 sequence containing mutations in both of the PXXP motifs. It is formally possible that these mutations may alter either the efficiency with which tyrosine 173 may be phosphorylated, or reduce the general accessibility of the phosphorylated motif to binding by SH2 domains. To take these possibilities into account, we also examined the relative efficiency of binding of a control CD28 binding partner which binds to the same phosphorylated motif. This control partner consists of the C terminal SH2 domain of human p85□. Since it does not have any SH3 domains, it would not be predicted to have the scope for specific interaction with the CD28 PXXP residues, and any perturbation of its binding by the mutations would most likely be due to such effects as described above. Any relative excess perturbation of GRIP binding by these mutations, over and above that seen for the control protein, is indicative of an active role played by the PXXP motifs, most likely through interaction with the GRIP SH3 domains.

These experiments were performed in the yeast two hybrid system. First, a PCR fragment containing the complete intracellular domain of human CD28 was constructed by PCR as for pCD28Y.BD, except that primer CC1208 was used in place of CC206 and CC1211 in place of CC207. The resulting PCR product was digested with BssHII and NotI, and cloned into a suitably prepared variant of pGEX4T3 that possesses AscI and NotI cloning sites. This vector was simply used to facilitate cloning and sequencing of the PCR product – any similar vector with single instances of these restriction sites could be used in the same role. AscI and BssHII restriction sites have compatible overhangs, such that a fragment cleaved with BssHII may be ligated into an AscI site, and subsequently liberated from this site by digestion with BssHII.

A clone of the correct structure was identified by fluorescent dye-terminator sequencing and the insert removed by digestion with BssHII and NotI. This fragment was then ligated into suitably prepared pAS1CYH2/BssHII and a

plasmid clone of the correct structure identified by sequencing. The resulting plasmid, pCD28PP.BD, encodes a GAL4BD-CD28 fusion protein in which the proline residues normally found at positions 178, 181, 190 and 193 of the mature human CD28 molecule are replaced by alanine residues.

Samples of plasmids pCD28Y.BD, pCD28F.BD and pCD28PP.BD were individually cotransfected into Y4.1lck yeast with samples of either pGRIPFL.AD or p85SH2C.AD plasmid. pCD28F.BD, which encodes a GAL4BD-CD28 fusion protein in which tyrosine 173 equivalent residue is replaced by phenylalanine. This fusion protein is therefore incapable of being phosphorylated at this site, and hence of supporting SH2 domain binding. The LacZ signals produced by the various GAL4BD fusion proteins in combination with this GAL4AD-CD28 fusion protein therefore define the background level of LacZ activity in the experiment.

Transformants were selected on media deficient in tryptophan and leucine as described (Fuller et al, 1998), and single colonies grown up in liquid culture in Met- media to induce the Lck gene. To analyse the amount of LacZ activity induced by the interaction of the two hybrid fusion partners, we employed a quantitative liquid assay similar to that described by Harshman et al (1988). 1 ml samples of mid-log phase cultures were harvested by centrifugation and resuspended in 100 mM Tris pH7.5/0.05% Triton X100, 200 □l per sample. The yeast cells were lysed by two cycles of rapid freezing in liquid nitrogen followed by rapid thawing in a 37°C water bath. For each sample, a tube containing 600 □l of chromogen solution was prepared. Chromogen solution is a mixture of Z-buffer, ONPG solution and 2-mercaptoethanol mixed in the volume ratio 500:100:1.644. Z-buffer is 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 0.1 mM $MgSO_4$, pH7.0. ONPG solution is 4 mg/ml w/v o-nitrophenyl-□-D-galactopyranoside (Sigma Chemical Co) prepared in 100 mM sodium phosphate buffer pH7.5. The lysate samples were vortexed vigorously, and a fixed volume V transferred to the prepared tubes containing the chromogen solution. These were rapidly vortexed and transferred to a 37°C water bath where they were

incubated for a time T before the reaction was terminated by the addition of 0.25V 1M Na_2CO_3 solution. Each tube was then centrifuged and the absorbance of a sample of the supernatant measured at 420 nm, giving the value A. To allow normalisation of the amount of LacZ activity according to the number of cells in each culture, a volume of the lysate W □l was diluted in 1 ml water and the absorbance measured at 600 nm, giving value B. The activity U of LacZ was then obtained, calibrated in arbitrary units, according to the formula U = 1000 A W / B V T.

The findings from a representative experiment were as follows:

GAL4AD fusion	LacZ activity produced by interaction with GAL4BD fusion (units)		
	pCD28Y.BD	pCD28F.BD	pCD28PP.BD
pGRIPFL.AD	0.322	0.005	0.040
p85SH2C.AD	0.374	0.005	0.225

These data show that for GRIP, the amount of LacZ activity produced by the association with the PXXP mutant CD28 is only 12.4% of that produced by association with the wild-type CD28. For the p85SH2 domain, the proportion is 60%. These results may be interpreted as follows. While the mutations in the CD28PP construct do affect the efficiency of interaction with p85SH2, as assessed by LacZ activity induction, the same mutations affect the interaction with GRIP to a much greater degree, indicative of a contribution of the PXXP motifs to the total interaction efficiency. These results demonstrate that one or both of the CD28 PXXP motifs are required for optimal binding efficiency to GRIP, most likely through their association with one or both of GRIP's SH3 domains. In combination with our earlier results, these data demonstrate that

such associations are neither necessary nor sufficient for GRIP binding to CD28, but form a substantial contributory element.

It will be obvious therefore that agents which perturb the interactions between either or both of the GRIP SH3 domains and binding partner proteins like CD28, while they may not necessarily abrogate GRIP binding, may nevertheless substantially modulate the total binding efficiency. Such modulation may dramatically alter the nature or magnitude of the function performed by GRIP in any particular system, for example by altering the half-life of a complex between GRIP and a partner protein, or in the case where one or more other proteins compete with GRIP for a mutually exclusive binding site on a partner protein, by altering the relative balance between GRIP and these other proteins in binding to the partner protein. Such modulation may be therapeutically desirable in conditions where GRIP is over-active, or insufficiently active or displays an inappropriate type of activity, or where competing proteins display undesired activity. It will also be obvious that the type of system described here may readily be adapted and varied by one ordinarily skilled in the art in order to search for such agents by screening.

Example 12: GRIP is recruited to activated CD28 receptor in vivo

To investigate whether GRIP forms a physiological part of the CD28 signalling complex, CD28 receptor was activated by cross-linking with an activating antibody, immunoprecipitated, and the resulting co-precipitating proteins analysed by Western blotting.

16 x 10^7 Jurkat T cells were washed in culture medium lacking foetal calf serum (SFM), pelleted by centrifugation, resuspended in 4 ml of SFM, and divided into 2 equal volume aliquots. These were prewarmed to 37°C for 10 minutes and

then 20 □g of goat antibody to mouse Ig (cat M2650; Sigma Chemical Co) were added to each aliquot.

After a further two minutes incubation at 37°C, one aliquot of cells, labelled t=0, was lysed by the addition of 2 ml of 2 x RIPA (2% NP40, 1% sodium deoxycholate, 0.2% SDS in PBS + protease/phosphatase inhibitors: Complete cocktail; Boehringer-Mannheim cat ; made up to twice normal concentration + 2 mM sodium orthovanadate)) and incubated on ice for 15 minutes. After this lysis step, 5.6 □g of anti-CD28 antibody clone 9.3 (see for example Bjorndahl et al, 1989) were added. To the second aliquot of cells, labelled t=4, 5.6 □g of the anti-CD28 antibody were added and the cells incubated at 37°C for 4 minutes. They were then lysed as for the first aliquot.

Both samples were then centrifuged to remove insoluble matter, and then tumbled with 80 □l per sample of ProteinA/G agarose (Pierce) overnight at 4°C. After centrifugation, the supernatants were discarded and the resins in each sample were then washed extensively in 1 x RIPA (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS; + protease/phosphatase inhibitors: Complete cocktail made at normal concentration + 1 mM sodium orthovanadate). Bound proteins were then eluted from the resins by boiling for 5 minutes in SDS-PAGE sample buffer. Stripped resins were pelleted by centrifugation, and samples of the supernatant containing eluted proteins analysed by SDS-PAGE and Western blotting by standard means.

In one such experiment, a Western blot of these samples was performed using monoclonal antibody to GRIP clone 1-13.4. In addition to samples of the t=0 and t=4 preparations, the blot also contained samples of Jurkat lysate which had been prepared simply by lysing Jurkat T cells in 2 x RIPA and immediately adding SDS-PAGE sample buffer, without any immunoprecipitation manipulations. This sample provides an internal control for the Western blotting part of the experiment.

The results are shown in Figure 13. In addition to non-specific signal derived from the cross-reaction of the HRP-anti mouse Ig secondary antibody with the murine anti-CD28 used for the immunoprecipitation, there is also a specific band, at the same molecular weight as GRIP, which appears in the t=4 sample and not in the t=0 sample. These data demonstrate that GRIP is indeed specifically recruited to the activated CD28 receptor.

This assay may also form the basis of a diagnostic kit for the determination of whether CD28 expressed on a lymphocyte surface has been recently activated, as might occur at a higher level than normal in autoimmune diseases, or at a lower level than normal in cancer or other immunosuppressive conditions. In such a kit, patient T lymphocytes would be purified by standard methods, lysed using the lysis buffer described above, and then the CD28 molecules immunoprecipitated as described above. Antibodies against GRIP antibody would then be used to determine whether the precipitated CD28 was associated with GRIP.

Furthermore, these data also demonstrate that GRIP is recruited to CD28 when the receptor is activated. Since therapies and manipulations which antagonise the CD28 signal are efficacious in autoimmune diseases, as demonstrated by in vivo experiments in models of rheumatoid arthritis, lupus, graft-versus-host-disease, transplant rejection, inflammatory bowel disease, multiple sclerosis, psoriasis, allergic asthma and contact dermatitis for example, it is to be expected that interventions which inhibit the function of GRIP will be similarly useful. Moreover, in diseases like cancer and immunosuppression where T cells are insufficiently activating, the provision of a CD28 signal, perhaps through the use of stimulating antibodies, has beneficial effects. In such states, interventions which promote the function of GRIP, perhaps by increasing its binding to CD28, or perhaps by increasing the efficiency with which it recruits partner proteins, may also have beneficial effect.

Example 13: GRIP associates with other signalling molecules

We also investigated the association of GRIP with other signalling proteins in order to elucidate the nature and identity of proteins that it might serve to recruit to activated CD28 receptor. 4 x 10^7 Jurkat cells were washed in culture medium lacking foetal calf serum (SFM), pelleted by centrifugation, resuspended in 1 ml SFM and lysed by the addition of 1 ml of 2 x RIPA. After incubation on ice for approximately 30 minutes, insoluble matter was removed by centrifugation. The resulting clarified lysate was tumbled at 4°C for 1 hour with 2.66 ml of glutathione sepharose 4B resin (Pharmacia) (previously washed in PBS according to the manufacturer's instructions and then equilibrated into 1x RIPA). The resin and any bound protein were removed by centrifugation, and the remaining supernatant (precleared lysate) divided into four equal volume aliquots.

Samples of glutathione sepharose 4B resin charged with either GST-GRIPFL, GST-GRIPSH2, GST-GRIPINS or GST were prepared as described above. Each sample of charged resin was mixed with one aliquot of precleared lysate and tumbled overnight at 4°C. The resin was pelleted by centrifugation and the supernatants discarded. After extensive washing of the resin with 1 x RIPA, bound proteins were eluted by boiling the resin in 100 □l of SDS-PAGE sample buffer for 5 minutes. Stripped resins were pelleted by centrifugation, and samples of the supernatant containing eluted proteins analysed by SDS-PAGE and Western blotting by standard means.

In one such experiment, the eluted proteins were analysed for the presence of Sos2 using a Sos2-specific antiserum (Santa Cruz catalogue number SC258). A band of the expected molecular weight was observed only in the sample where Jurkat lysate had been exposed to GST-GRIPFL (Figure 14). These data indicate that the full length GRIP protein has the potential to specifically

associate with signalling proteins such as Sos2, and therefore to recruit these proteins to activated CD28 receptor. The absence of association between GST-GRIPSH2 or GST-GRIPINS and Sos2 implies that it is most likely one or both of the GRIP SH3 domains, present only in GST-GRIPFL, which mediates the particular interaction with Sos2. Similarly, GRIP may associate with other proteins by means of either or both of the SH3 domains, the SH2 domain or the insert domain, or combinations of these or fragments thereof.

Example 14: Development of a yeast assay for proteins that associate with GRIP

To facilitate the identification of proteins which associate with GRIP, a yeast two hybrid assay was developed. Plasmid pGRIP.BDI (see above) was linearised by digestion with XbaI and transfected into Saccharomyces cerevisiae Y190 (Harper et al, 1993). Transformed clones were identified by growth on tryptophan-deficient media, and single clonal colonies isolated by two rounds of streaking out of single colonies. Six such clones were grown up and chromosomal DNA purified as described (Fuller et al, 1998).

Two oligonucleotide primers were designed, one selectively hybridising to sequence encoding the GAL4BD protein, and the other selectively hybridising to the GRIP coding sequence, on the opposing strand to the first primer. These primers were so chosen that if used in PCR with template DNA derived from pGRIP.BDI, a product band of approximately 520 bp would be obtained. The primers were synthesised and employed in standard PCRs using samples of the yeast clone DNA preparations as template. DNA derived from one clone, termed Y190/pGRIP.BDI, produced the expected band, so this yeast clone was selected for further work.

Oligonucleotide primers CE130 and CASOS2 were designed to amplify cDNA encoding the final 197 amino acid residues of human Sos2 and to provide it with

cloning sites such that it could be ligated into pACT2/BamHI to form an in-frame fusion with GAL4AD. This region of Sos2 is rich in Pro-Xxx-Xxx-Pro motifs which may mediate interactions with SH3 containing proteins such as GRIP. The predicted cDNA sequence of Sos2 was produced by making a consensus sequence between the relevant portions of Genbank sequences L20686, AA621168 and H01561. These primers were employed in a RT-PCR using Ready-to-Go reagents according to the manufacturer's instructions (Pharmacia) and 4 □l of total RNA purified from Jurkat cells as described previously. Forty cycles of amplification were applied, and the resulting PCR product purified by means of the Wizard DNA clean up kit (Promega). A sample of this cDNA fragment was then used as template in a second PCR using primers CE130 and CASOS2. The product of this reaction was digested with BamHI and XhoI and ligated into suitably prepared pACT2/BamHI. A number of clones were analysed by restriction digestions diagnostic for the presence of the Sos2 insert, and one positive clone selected for further analysis. This clone was analysed by fluorescent dye-terminator sequencing, and found to have the predicted sequence. This plasmid, pACT2/Sos2, therefore encodes a GAL4AD-Sos2 fusion protein.

Samples of pACT2/Sos2 and parental pACT2/BamHI plasmid DNA were transformed into either yeast Y190/pGRIP.BDI or parental Y190 according to standard protocols. Transformants were selected for growth on media deficient in tryptophan and leucine or leucine only, as appropriate, and subsequently analysed for LacZ activity as described (Fuller et al, 1998). The results are shown in the table below, scored according to the amount of LacZ activity seen. – indicates no LacZ activity, + indicates trace amounts of LacZ activity, ++, +++ and ++++ indicate progressively stronger amounts of LacZ activity.

Plasmid	Yeast strain	
	Y190/pGRIP.BDI	Y190
pACT2/Sos2	++++	-
pACT2	+	Not done

These data indicate that GRIP interacts specifically with the C terminal fragment of Sos2 and that this interaction can be assayed in the yeast two-hybrid format. Since in this yeast system there is no exogenously supplied protein tyrosine kinase, these results also demonstrate that GRIP is capable of forming non-phosphotyrosine-dependent associations with other signalling proteins.

It will be readily apparent to one skilled in the art that similar yeast two hybrid assays may be derived from this work by application of well known molecular biological techniques and applied to the discovery of novel proteins which interact with some part of GRIP protein. It will also be apparent that similar assays may be employed to identify by screening agents which specifically modulate the association of GRIP polypeptides with previously identified protein binding partners. One such agent may be the portion of Sos2 employed above, or peptides or fragments derived therefrom.

Example 15: Screening for inhibitors of the GRIP-CD28 interaction

To provide a convenient assay for the screening of inhibitors of the interaction of GRIP with CD28, an assay was prepared in which the binding of GST – GRIPFL to peptides derived from the portion of the CD28 cytoplasmic domain could be modulated. A single peptide corresponding to the CD28 sequence around the tyrosine 173 was chemically synthesised. The peptide sequence was [biotin]-

KLLHSDpYMNMT where [biotin]-K indicates a lysyl residue bearing a biotin moiety and pY indicates a phosphotyrosine residue.

Nunc Maxisorp microtitre plates were coated with 2 μg/ml of streptavidin (STAR1B; Serotec; Kidlington; UK) in PBS, 100 μl per well, and stored overnight at 4°C. After washing with TBS/0.1% Tween 20, unoccupied protein binding sites on the plate were blocked by incubation with 200 μl per well of a 3% w/v solution of BSA in PBS overnight at 4°C. After further washing as above wells were exposed to an approximate 5 μM solution of peptide ('bound peptide') in PBS, 100 μl per well for approximately 1 hour at room temperature. Plates were washed again and then exposed to approximately equal volumes of 0.156-10μM peptide ('inhibitory peptide') in PBS and a solution of GST-GRIPFL in PBS, in a total volume of 100 μl per well for approximately 1 hour at room temperature. Plates were washed again and then exposed to a 1/2000 v/v solution of goat anti-GST antiserum (Pharmacia) in PBS, 100 μl per well for approximately 1 hour at room temperature. Plates were washed again and then exposed to a horseradish peroxidase- conjugated antiserum directed against goat Ig (A5420; Sigma Chemical Co) at a concentration of 1/2000 v/v in PBS, 100 μl per well for approximately 1 hour at room temperature. Plates were washed for a final time and the bound peroxidase activity quantitated by use of a chromogenic substrate (Fast OPD; Sigma Chemical Co) according to the manufacturer's instructions. After the chromogenic reaction had proceeded to an appropriate extent, it was terminated by the addition of 3M sulphuric acid, 25 μl per well, and the amount of reaction product quantitated by determining the absorbance at 490 nm.

In this assay, GST-GRIPFL showed a specific interaction with the bound peptide. The presence of increasing concentrations of inhibitory peptide (0.156μM-10μM) inhibited the association of GST-GRIPFL with bound peptide, such that at the presence of 5μM and 10μM inhibitory peptide caused a reduction of 32% and

60% respectively in the amount of GST-GRIPFL associated with bound CD28 peptide.

It will be readily apparent to one skilled in the art that such simple biochemically defined assays may be easily adapted and utilised to screen for agents that specifically modulate the interaction between full length GRIP or fragments of GRIP and CD28 or other proteins or peptides derived therefrom.

References

Allison, J.P. 1994. CD28-B7 interactions in T-cell activation. *Curr Opin Immunol 6:414.*

Azuma, M., D. Ito, H. Yagita, K. Okumura, J.H. Phillips, L.L Lanier and C. Somoza. 1993. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature 366:76.*

Barclay, A.N., M.H. Brown, S.K.A. Law, A.J. McKnight. M.G. Tomlinson and P.A. van der Merwe. 1997. The Leukocyte Antigen Factsbook. 2nd ed. Academic Press: London, UK.

Birge, R.B., B.S. Knudsen, D. Besser and H. Hanafusa. 1996. SH2 and SH3-containing adaptor proteins: redundant or independent mediators of intracellular signal transduction. *Genes to Cells 1:595.*

Bjorndahl, J.M., S.S. Sung, J.A. Hansen and S.M. Fu. 1989. Human T cell activation: differential response to anti-CD28 as compared to anti-CD3 monoclonal antibodies. *Eur J Immunol 19:881.*

Caux, C., B. Vanbervliet, C. Massacrier, M. Azuma, K. Okumura, L. Lanier and J. Banchereau. 1994. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J Exp Med 180:1841.*

Corry, D.B., S.L. Reiner, P.S. Linsley and R.M. Locksley. 1994. Differential effects of blockade of CD28-B7 on the development of Th1 or Th2 effector cells in experimental leishmaniasis. *J Immunol 153:4142.*

DeSilva, D.R., K.B. Urdahl and M.K. Jenkins. 1991. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J Immunol 147:3261.*

Ellis, J.H., M.N. Burden, D.V. Vinogradov, C. Linge and J.S. Crowe. 1996. Interactions of CD80 and CD86 with CD28 and CTLA4. *J Immunol 156:2700.*

Feng, G.S., Y.B. Ouyang, D.P. Hu, Z.Q. Shi, R. Gentz and J. Ni. 1996. Grap is a novel SH3-SH2-SH3 adaptor protein that couples tyrosine kinases to the Ras pathway. *J Biol Chem 271:12129.*

Finck, B.K., P.S. Linsley and D. Wofsy. 1994. Treatment of murine lupus with CTLA4Ig. *Science 265:1225.*

Freeman, G.J., A.S. Freedman, J.M. Segil, G. Lee, J.F. Whitman and L.M. Nadler. 1989. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J Immunol 143:2714.*

Freeman, G.J., G.S. Gray, C.D. Gimmi, D.B. Lombard, L.J. Zhou, M. White, J.D. Fingeroth, J.G. Gribben and L.M. Nadler. 1991. Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J Exp Med 174:625.*

Freeman, G.J., F. Borriello, R.J. Hodes, H. Reiser, J.G. Gribben, J.W. Ng, J. Kim, J.M. Goldberg, K. Hathcock, G. Laszlo, L.A. Lombard, S. Wang, G.S. Gray, L.M. Nadler and A.H. Sharpe. 1993a. Murine B7-2, an alternative CTLA4 counter-receptor that costimulates T cell proliferation and interleukin 2 production. *J Exp Med 178:2185.*

Freeman, G.J., J.G. Gribben, V.A. Boussiotis, J.W. Ng, V.A. Restivo, L.A. Lombard, G.S. Gray and L.M. Nadler. 1993b. Cloning of B7-2; a CTLA4 counter-receptor that costimulates human T cell proliferation. *Science 262:909.*

Fuller, K.J., M.A. Morse, J.H.M. White, S.J. Dowell and M.J. Sims. 1998. Development of a yeast trihybrid screen using stable yeast strains and regulated protein expression. *Biotechniques 25:85.*

Galvin, F., G.J. Freeman, Z. Razi-Wolf, W.J. Hall, B. Benacerraf, L. Nadler and H. Reiser. 1992. Murine B7 antigen provides a sufficient costimulatory signal for antigen-specific and MHC restricted T cell activation. *J Immunol 149:3802.*

Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raulet, J.P. Allison. 1992. CD28-mediated signalling costimulates murine T cells and prevents induction of anergy in T cell clones. *Nature 356:607.*

Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi and S.J. Elledge. 1993. the p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell 75:805.*

Harshman, K.D., W.S. Moye-Rowley and C.S. Parker. 1988. Transcriptional activity by the SV40 AP-1 recognition element in yeast is mediated by a factor similar to AP-1 that is distinct from GCN4. *Cell 53:321.*

Judge, T.A., M. Liu, P.J. Christensen, J.J. Fak and L.A. Turka. 1995. Cloning the rat homolog of the CD28/CTLA4-ligand B7-1: structural and functional analysis. *Int Immunol 7:171.*

June, C.H., J.A. Ledbetter, P.S. Linsley and C.B. Thompson. 1990. Role of CD28 receptor in T cell activation. *Immunol Today 11:211.*

June, C.H., P. Vandenburghe and C.B. Thompson. 1994a. The CD28 and CTLA-4 receptor family. *Chem Immunol 59:62.*

June, C.H., J.A. Bluestone, L.M. Nadler and C.B. Thompson. 1994b. The B7 and CD28 receptor families. *Immunol Today 15:321.*

King, P.D., A. Sadra, J.M. Teng, L. Xiao-Rong, A. Han, A. Selvakumar, A. August and B. Dupont. 1997. Analysis of CD28 cytoplasmic tail tyrosine residues as regulators and substrates for the protein tyrosine kinases EMT and LCK. *J Immunol 158:580.*

Katayama, I., T. Matsunaga, H. Yokozeki and K. Nishioka. 1997. Blockade of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) down-regulates induction of contact sensitivity by haptenated epidermal cells. *Br J Dermatol 136:846.*

Keane-Myers, A.M., W.C. Gause, F.D. Finkelman, X.D. Xhou and M. Wills-Karp. 1998. Development of murine allergic asthma is dependent upon B7-2 costimulation. *J Immunol 160:1036.*

Kilpatrick, K.E., S.A. Wring, D.H. Walker, M.D. Macklin, J.A. Payne, J.L. Su, B.R. Champion, B. Caterson and G.D. Mcintyre. 1997. Rapid development of affinity matured monoclonal antibodies using RIMMS. *Hybridoma 16:381.*

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucl Acids Res 12:857.*

Leach, D.R., M.F. Krummel and J.P. Allison. 1996. Enhancement of anti-tumour immunity by CTLA4 blockade. *Science 271:1734.*

Ledbetter, J.A., J.B. Imboden, G.L. Schieven, L.S. Grosmaire, P.S. Rabinovitch, T. Lindsten, C.B. Thompson and C.H. June. 1990. CD28 ligation in T cell activation: evidence for two signal transduction pathways. *Blood 75:1531.*

Linsley, P.S., W. Brady, L. Grosmaire, A. Aruffo, N.K. Damle and J.A. Ledbetter. 1991a. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med 173:721.*

Linsley, P.S., W. Brady, M. Urnes, L.S. Grosmaire, N.K. Damle and J.A. Ledbetter. 1991b. CTLA4 is a second receptor for the B cell activation antigen B7. *J Exp Med 174:561.*

Linsley, P.S., P.M. Wallace, J. Johnson, M.G. Gibson, J.L. Greene, J.A. Ledbetter, C. Singh and M.A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science 257:792.*

Lowenstein, E.J., R.J. Daly, A.G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E.Y. Skolnik, D. Bar-Sagi and J Schlessinger. 1992. The SH2 and SH3 domain-containing protein Grb2 links receptor tyrosine kinases to ras signalling. *Cell 70:431.*

Marengere, L.E., P. Waterhouse, G.S. Duncan, H.W. Mittrucker, G.S. Feng and T.W. Mak. 1996. Regulation of T cell receptor signalling by tyrosine phosphatase Syp assocation with CTLA4. *Science 272:1170.*

Mueller, D.L., M.K. Jenkins and R.H. Schwartz. 1989a. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J Immunol 142:2617.*

Mueller, D.L., M.K. Jenkins and R.H. Schwartz. 1989b. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Ann Rev Immunol 7:445.*

Musacchio, A., M. Wilmanns and M. Saraste. 1994. Structure and function of the SH3 domain. *Prog Biophys Mol Biol 61:283.*

Peach, R.J., J. Bajorath, W. Brady, G. Leytze, A. Greene, J. Naemura and P.S. Linsley. 1994. Complementarity determining region 1 (CDR1)- and CDR3-analogous regions in CTLA4 and CD28 determine the binding to B7-1. *J Exp Med 180:2049.*

Peri, K.G. and A. Veillette. 1994. Tyrosine protein kinases in T lymphocytes. *Chem Immunol 59:19.*

Perrin, P.J., D. Scott, L. Quigley, P.S. Albert, O. Feder, G.S. Gray, R. Abe, C.H. June and M.K. Racke. 1995. Role of B7:CD28/CTLA4 in the induction of chronic relapsing experimental allergic encephalomyelitis. *J Immunol 154:1481.*

Quill, H. and Schwartz, R.H. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative non-responsiveness. *J Immunol 138:3704.*

Raab, M., Y.C. Cai, S.C. Bunnell, S.D. Heyeck, L.J. Berg and C.E. Rudd. 1995. p56Lck and p59Fyn regulate CD28 binding to phosphatidylinositol 3-kinase, growth factor receptor-bound protein GRB2 and T cell specific protein tyrosine

kinase ITK: implications for T cell costimulation. *Proc Natl Acad Sci USA 92:8891.*

Ronchese, F., B. Hausmann, S. Hubele and P. Lane. 1994. Mice transgenic for a soluble form of murine CTLA4 show enhanced expansion of antigen-specific CD4-positive T cells and defective antibody production in vivo. *J Exp Med 179:809.*

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular cloning: A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, US.

Schaffhausen, B. 1995. SH2 domain structure and function. *Biochim Biophys Acta 1242:61.*

Schnieder, H., K.V. Prasad, S.E. Shoelson and C.E. Rudd. 1995. CTLA4 binding to the lipid kinase phosphatidylinositol 3-kinase in T cells. *J Exp Med 181:351.*

van Seventer, G.A., W. Newman, Y. Shimizu, T.B. Nutman, Y. Tanaka, K.J. Horgan, Y.V. Gopal, E. Ennis, D. O'Sullivan, H. Grey and S. Shaw. 1991. Analysis of T cell stimulation by superantigen plus major histocompatibility complex class II molecules or by CD3 monoclonal antibody: costimulation by purified adhesion ligands VCAM-1, ICAM-1 but not ELAM-1. *J Exp Med 174:901.*

Wallace, P.M., J.S. Johnson, J.F. MacMaster, K.A. Kennedy, P. Gladstone and P.S. Linsley. 1994. CTLA4Ig treatment ameliorates the lethality of murine graft-versus-host disease across major histocompatibility complex barriers. *Transplantation 58:602.*

Walunas, T.L., C.Y. Bakker and J.A. Bluestone. 1996. CTLA4 ligation blocks CD28-dependent T cell activation. *J Exp Med 183:2541.*

Ward, S.G. 1996. CD28 – a signalling perspective. *Biochem J 318:361.*

Weiss, A. and J.B. Imboden. 1987. Cell surface molecules and early events involved in human T lymphocyte activation. *Adv Immunol 41:1.*

Zhang, Y. and J.P. Allison. 1997. Interaction of CTLA4 with AP50, a clathrin-coated pit adaptor protein. *Proc Natl Acad Sci USA 94:9273.*

Claims

1. A polypeptide comprising the amino acid sequence shown in Figure 5 or any fragment thereof containing at least the amino acid residues encoded by nucleotide residues 151-459 or any polypeptide having substantially the same sequence and capable of binding to human CD28.

2. A polypeptide according to claim 1 wherein the polypeptide has a sequence that is at least 80% homologous to that of Figure 5.

3. A polypeptide according to claim 1 wherein the polypeptide has sequence that is at least 95% homologous.

4. A polypeptide according to claim 1 wherein the polypeptide has sequence that is at least 99% homologous.

5. A polypeptide according to any of the above claims which is capable of binding to CD28 at or near phosphorylated tyrosine 173.

6. A polypeptide according to any of the above claims which is attached to a carrier molecule.

7. A method of preventing a polypeptide according to any of the above claims binding to human CD28 comprising the use of a compound which is capable of inhibiting such binding.

8. A method according to claim 7 wherein the compound which is capable of inhibiting binding between CD28 and said polypeptide is selected from an antibody, antibody derivatives, peptides, phosphorylated peptides or aptamers.

9. A method of identifying a compound that inhibits binding between CD28 and a polypeptide according to any of claims 1 to 6, which comprises use of a polypeptide according to any of claims 1 to 6 to screen for compounds that bind to said polypeptide.

10. A method of identifying a compound that inhibits binding between CD28 and a polypeptide according to any of claims 1 to 6 which comprises use of CD28 to screen for compounds that will bind to CD28 at or near phosphorylated tyrosine 173.

11. A method of treating a human patient with a disorder involving CD28-expressing cells comprising administering to the patient a compound that inhibits the binding of a polypeptide according to any of claims 1 to 6 to CD28.

12. A method according to claim 11 wherein said disorder is an autoimmune disorder or cancer.

13. A DNA sequence encoding a polypeptide according to any of claims 1 to 6.

Fig. 1

Polylinker of pYTH9/BssHII

```
      SalI        NcoI      ClaI     EcoRI   PstI           BssHII
GCT-AGG-TCG-ACG-GCC-ATG-GTA-TCG-ATG-AAT-TCC-TGC-AGC-CCG-GCG-CGC-TCT-
A   R   S   T   A   M   V   S   M   N   S   C   S   P   A   R   S

        SpeI    NotI
GGA-TCT-ACT-AGT-GCG-GCC-GCC-ACC-GCG-GTG
G   S   T   S   A   A   A   T   A   V
```

Fig. 2

Polylinker of pAS1CYH2/BssHII

```
NdeI SfiI NcoI                  BssHII       BamHI   SalI
CAT-ATG-GCC-ATG-GAG-GCC-CCG-GCG-CGC-TCT-GGA-TCC-GTC-GAC-CTG-CAG-CCA-
H   M   A   M   E   A   P   A   R   S   G   S   V   D   L   Q   P

AGC-TAA
S   Stop
```

09/763024
PCT/GB99/02738

Fig. 3

Polylinker of pACT2/BamHI

```
NdeI SfiI NcoI          SmaI            BamHI           EcoRI
CAT-ATG-GCC-ATG-GAG-GCC-CCG-GGG-ATC-GGA-TCC-GAT-CCG-AAT-TCG-AGC-TCG
H   M   A   M   E   A   P   G   I   G   S   D   P   N   S   S   S

XhoI
AGA-GAT-CTA-TGA
R   D   L   Stop
```

Fig. 4

Construction of artificial gene encoding the CD28 cytoplasmic domain in a form suitable for expression in the yeast two hybrid vectors. The domain is built up from overlapping primers (underlined) according to the scheme below:

```
     BssHII
   catcgcgcgcAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCACG
 1 ---------+---------+---------+---------+---------+---------+---------+ 60
   gtagcgcgcgTCATTCTCCTCGTCCGAGGACGTGTCACTGATGTACTTGTACTGAGGTGC

      A  R  S  K  R  S  R  L  L  H  S  D  Y  M  N  M  T  P  R  -

   TAGACCGGGTCCAACGAGAAAGCATTACCAGCCCTATGCACCACCTAGAGACTTCGCAGC
61 ---------+---------+---------+---------+---------+---------+---------+ 120
   ATCTGGCCCAGGTTGCTCTTTCGTAATGGTCGGGATACGTGGTGGATCTCTGAAGCGTCG

    R  P  G  P  T  R  K  H  Y  Q  P  Y  A  P  P  R  D  F  A  A  -

                  NotI    BssHII
   CTATCGCTCCTGAgcggccgcagcgcgcgatg
121 ---------+---------+---------+- 152
   GATAGCGAGGACTcgccggcgtcgcgcgctac

    Y  R  S  *
```

Two GC-rich stretches in the sequence have been recoded using the redundancy of the genetic code to give a more balanced base composition while retaining the same amino acid coding sequence. The construct includes restriction sites (bold): BssHII and NotI. The sequence includes only 1 extra residue not part of CD28 - the N-terminal Ala (double-underlined).

Fig. 5 Coding sequence of human GRIP: cDNA and deduced amino acid sequence

```
          ATGGAAGCTGTTGCCAAGTTTGATTTCACTGCTTCAGGTGAGGATGAACTGAGCTTTCAC
        1 ---------+---------+---------+---------+---------+---------+ 60
          TACCTTCGACAACGGTTCAAACTAAAGTGACGAAGTCCACTCCTACTTGACTCGAAAGTG

a         M  E  A  V  A  K  F  D  F  T  A  S  G  E  D  E  L  S  F  H   -

          ACTGGAGATGTTTTGAAGATTTTAAGTAACCAAGAGGAGTGGTTTAAGGCGGAGCTTGGG
       61 ---------+---------+---------+---------+---------+---------+ 120
          TGACCTCTACAAAACTTCTAAAATTCATTGGTTCTCCTCACCAAATTCCGCCTCGAACCC

a         T  G  D  V  L  K  I  L  S  N  Q  E  E  W  F  K  A  E  L  G   -

          AGCCAGGAAGGATATGTGCCCAAGAATTTCATAGACATCCAGTTTCCCAAATGGTTTCAC
      121 ---------+---------+---------+---------+---------+---------+ 180
          TCGGTCCTTCCTATACACGGGTTCTTAAAGTATCTGTAGGTCAAAGGGTTTACCAAAGTG

a         S  Q  E  G  Y  V  P  K  N  F  I  D  I  Q  F  P  K  W  F  H   -

          GAAGGCCTCTCTCGACACCAGGCAGAGAACTTACTCATGGGCAAGGAGGTTGGCTTCTTC
      181 ---------+---------+---------+---------+---------+---------+ 240
          CTTCCGGAGAGAGCTGTGGTCCGTCTCTTGAATGAGTACCCGTTCCTCCAACCGAAGAAG

a         E  G  L  S  R  H  Q  A  E  N  L  L  M  G  K  E  V  G  F  F   -

          ATCATCCGGGCCAGCCAGAGCTCCCCAGGGGACTTCTCCATCTCTGTCAGGCATGAGGAT
      241 ---------+---------+---------+---------+---------+---------+ 300
          TAGTAGGCCCGGTCGGTCTCGAGGGGTCCCCTGAAGAGGTAGAGACAGTCCGTACTCCTA
```

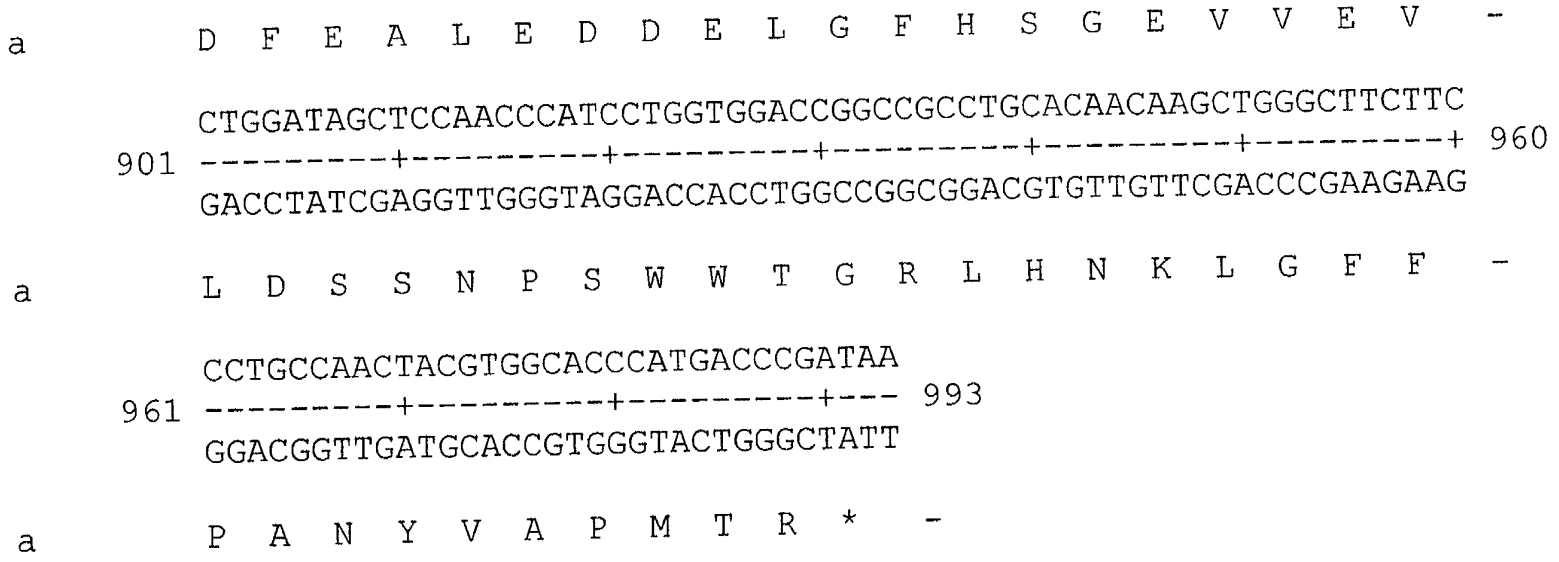
Fig. 5 cont.

```
a    I  I  R  A  S  Q  S  S  S  P  G  D  D  F  S  I  S  V  R  H  E  D  -

     GACGTTCAACACTTCAAGGTCATGCGAGACAACAAGGGTAATTACTTTCTGTGGACTGAG
301  ---------+---------+---------+---------+---------+---------+ 360
     CTGCAAGTTGTGAAGTTCCAGTACGCTCTGTTGTTCCCATTAATGAAAGACACCTGACTC

a    D  V  Q  H  F  K  V  M  R  D  N  K  G  N  Y  F  L  W  T  E  -

     AAGTTTCCTTCCCTAAATAAGCTGGTAGACTACTACAGGACAAATTCCATCTCCAGACAG
361  ---------+---------+---------+---------+---------+---------+ 420
     TTCAAAGGAAGGGATTTATTCGACCATCTGATGATGTCCTGTTTAAGGTAGAGGTCTGTC

a    K  F  P  S  L  N  K  L  V  D  Y  Y  R  T  N  S  I  S  R  Q  -

     AAGCAGATCTTCCTTAGAGACAGAACCCGAGAAGACCAGGGTCACCGGGGCAACAGCCTG
421  ---------+---------+---------+---------+---------+---------+ 480
     TTCGTCTAGAAGGAATCTCTGTCTTGGGCTCTTCTGGTCCCAGTGGCCCCGTTGTCGGAC

a    K  Q  I  F  L  R  D  R  T  R  E  D  Q  G  H  R  G  N  S  L  -

     GACCGGAGGTCCCAGGGAGGCCCACACCTCAGTGGGGCTGTGGGAGAAGAAATCCGACCT
481  ---------+---------+---------+---------+---------+---------+ 540
     CTGGCCTCCAGGGTCCCTCCGGGTGTGGAGTCACCCCGACACCCTCTTCTTTAGGCTGGA

a    D  R  R  S  Q  G  G  P  H  L  S  G  A  V  G  E  E  I  R  P  -

     TCGATGAACCGGAAGCTGTCGGATCACCCCCCGACCCTTCCCCTGCAGCAGCACCAGCAC
541  ---------+---------+---------+---------+---------+---------+ 600
     AGCTACTTGGCCTTCGACAGCCTAGTGGGGGGCTGGGAAGGGGACGTCGTCGGTGGTCGTG
```

Fig. 5 cont.

```
a       S  M  N  R  K  L  S  D  H  P  P  T  L  P  L  Q  Q  H  Q  H  -

        CAGCCACAGCCTCCGCAATATGCCCCAGCGCCCCAGCAGCTGCAGCAGCCCCCACAGCAG
601     ---------+---------+---------+---------+---------+---------+ 660
        GTCGGTGTCGGAGGCGTTATACGGGGTCGCGGGGTCGTCGACGTCGTCGGGGGTGTCGTC

a       Q  P  Q  P  P  Q  Y  A  P  A  P  Q  Q  L  Q  Q  P  P  Q  Q  -

        CGATATCTGCAGCACCACCATTTCCACCAGGAACGCCGAGGAGGCAGCCTTGACATAAAT
661     ---------+---------+---------+---------+---------+---------+ 720
        GCTATAGACGTCGTGGTGGTAAAGGTGGTCCTTGCGGCTCCTCCGTCGGAACTGTATTTA

a       R  Y  L  Q  H  H  H  F  H  Q  E  R  R  G  G  S  L  D  I  N  -

        GATGGGCATTGTGGCACCGGCTTGGGCAGTGAAATGAATGCGGCCCTCATGCATCGGAGA
721     ---------+---------+---------+---------+---------+---------+ 780
        CTACCCGTAACACCGTGGCCGAACCCGTCACTTTACTTACGCCGGGAGTACGTAGCCTCT

a       D  G  H  C  G  T  G  L  G  S  E  M  N  A  A  L  M  H  R  R  -

        CACACAGACCCAGTGCAGCTCCAGGCGGCAGGGCGAGTGCGGTGGGCCCGGGCGCTGTAT
781     ---------+---------+---------+---------+---------+---------+ 840
        GTGTGTCTGGGTCACGTCGAGGTCCGCCGTCCCGCTCACGCCACCCGGGCCCGCGACATA

a       H  T  D  P  V  Q  L  Q  A  A  G  R  V  R  W  A  R  A  L  Y  -

        GACTTTGAGGCCCTGGAGGATGACGAGCTGGGGTTCCACAGCGGGGAGGTGGTGGAGGTC
841     ---------+---------+---------+---------+---------+---------+ 900
        CTGAAACTCCGGGACCTCCTACTGCTCGACCCCAAGGTGTCGCCCCTCCACCACCTCCAG
```

Fig. 5 cont.


a D F E A L E D D E L G F H S G E V V E V -
CTGGATAGCTCCAACCCATCCTGGTGGACCGGCCGCCTGCACAACAAGCTGGGCTTCTTC
901 ---------+---------+---------+---------+---------+---------+ 960
GACCTATCGAGGTTGGGTAGGACCACCTGGCCGGCGGACGTGTTGTTCGACCCGAAGAAG
a L D S S N P S W W T G R L H N K L G F F -
CCTGCCAACTACGTGGCACCCATGACCCGATAA
961 ---------+---------+---------+--- 993
GGACGGTTGATGCACCGTGGGTACTGGGCTATT
a P A N Y V A P M T R * -

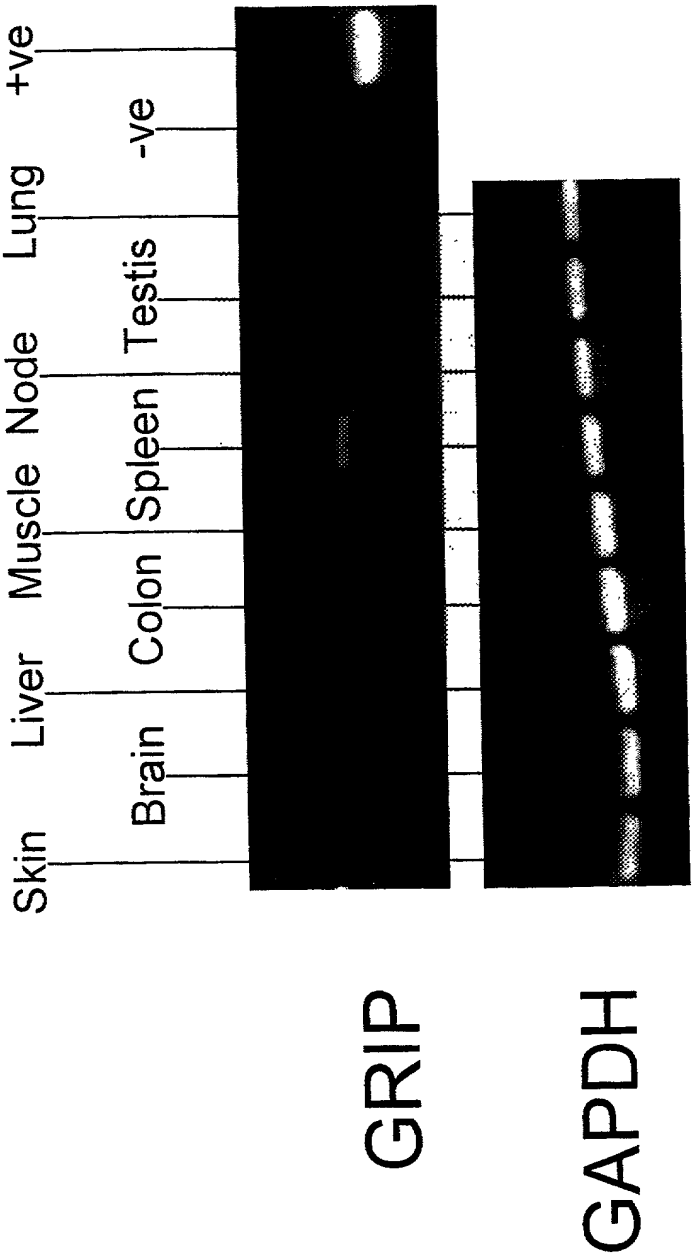


Fig. 6

Tissue expression of GRIP mRNA.

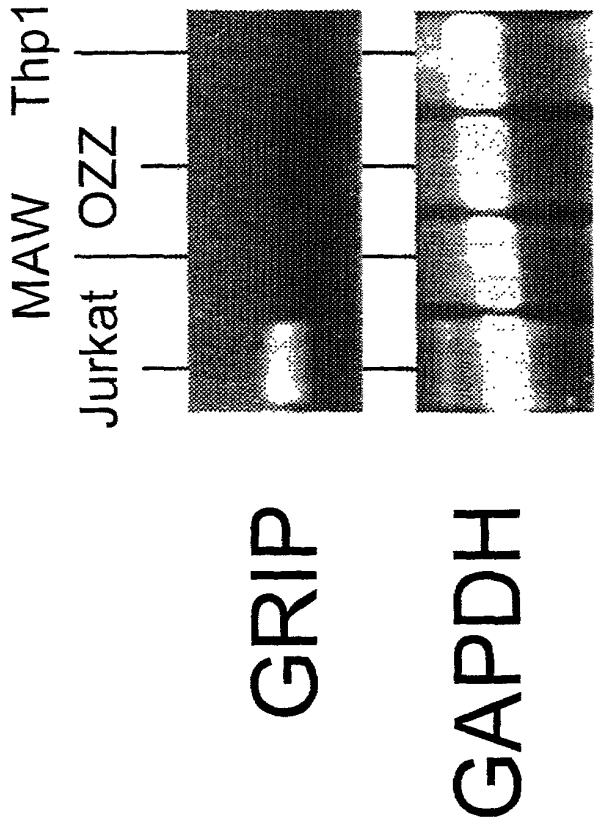


Fig. 7

Expression of GRIP mRNA in cell lines

Fig. 8

Modulation of GRIP mRNA levels by cell activation

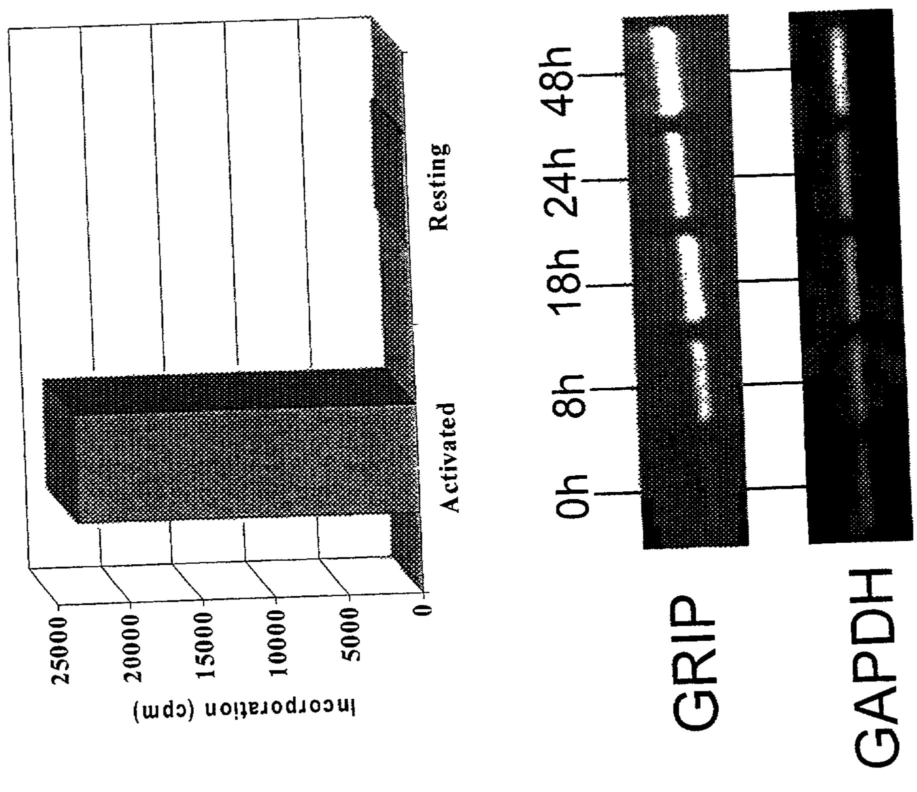


Fig. 9

Junction sequences in pGRIPFL.FIX. GRIP sequences are underlined.

```
    AGCTTACCATGGGGGGTTCTCATCATCATCATCATGGTATGGCTAGCATGACTGGTG
1   ---------+---------+---------+---------+---------+---------+   60
    TCGAATGGTACCCCCCAAGAGTAGTAGTAGTAGTAGTACCATACCGATCGTACTGACCAC
            M   G   G   S   H   H   H   H   H   H   G   M   A   S   M   T   G   G

    GACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGTCTAGAGGATCCAAGCTTA
61  ---------+---------+---------+---------+---------+---------+  120
    CTGTCGTTTACCCAGCCCTAGACATGCTGCTACTGCTATTCAGATCTCCTAGGTTCGAAT
      Q   Q   M   G   R   D   L   Y   D   D   D   D   K   S   R   G   S   K   L   M

    TGGAAGCTGTTGCCAAGTTTGATTTCACTGCTTCAGGTGAGGATGAACTGAGCTTTCACA
121 ---------+---------+---------+---------+---------+---------+  180
    ACCTTCGACAACGGTTCAAACTAAAGTGACGAAGTCCACTCCTACTTGACTCGAAAGTGT
      E   A   V   A   K   F   D   F   T   A   S   G   E   D   E   L   S   F   H   T

    CTGGAGATGTTTTGAAGATTTTAAGTAACCAAGAGGAGTGGTTTAAGGCGGAGCTTGGGA
181 ---------+---------+---------+---------+---------+---------+  240
    GACCTCTACAAAACTTCTAAAATTCATTGGTTCTCCTCACCAAATTCCGCCTCGAACCCT
      G   D   V   L   K   I   L   S   N   Q   E   E   W   F   K   A   E   L   G
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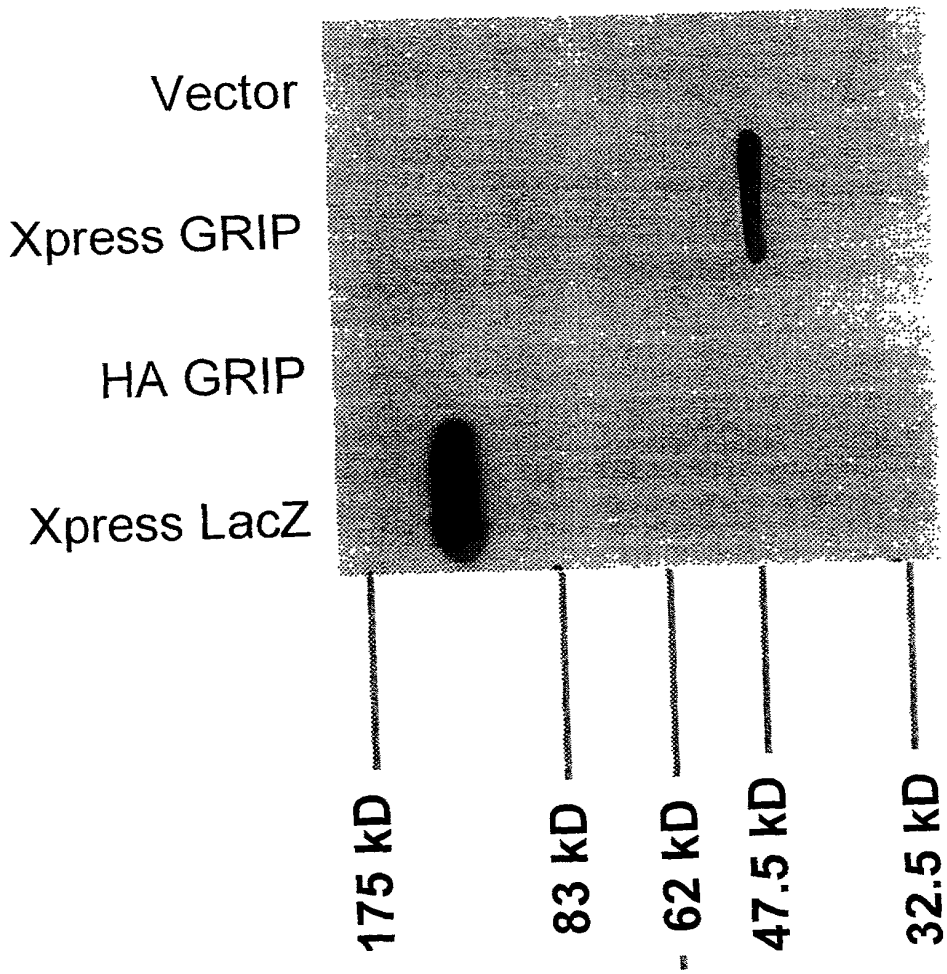


Fig. 10

Expression of Xpress-tagged GRIP in eukaryotic cells.

Fig. 11

Recognition of natively expressed GRIP from Jurkat cells by anti-GRIP monoclonal antibody 1-13.4.

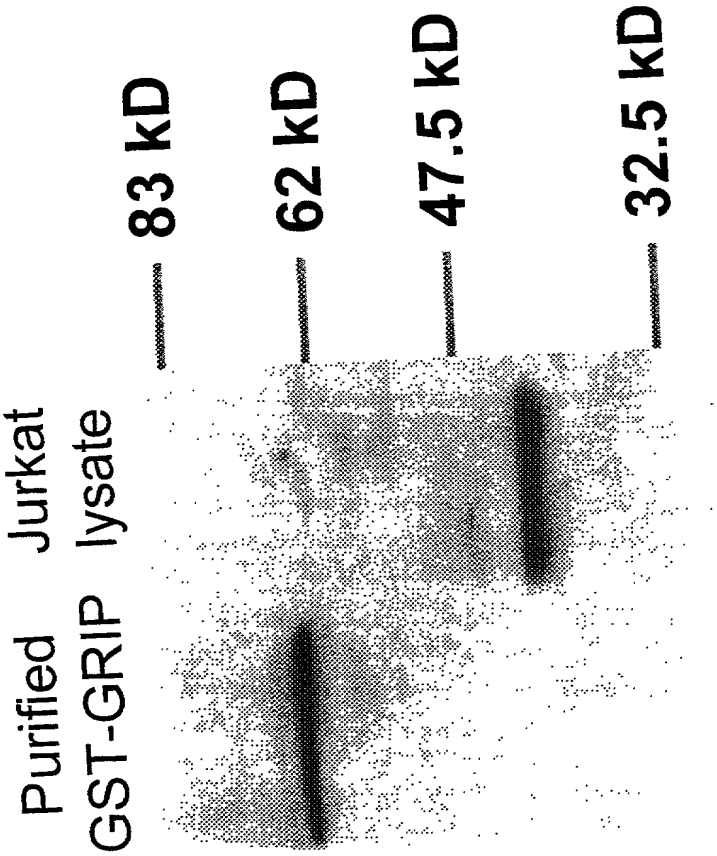


Fig. 12

Binding of GST-GRIP fusion proteins to CD28 peptides.

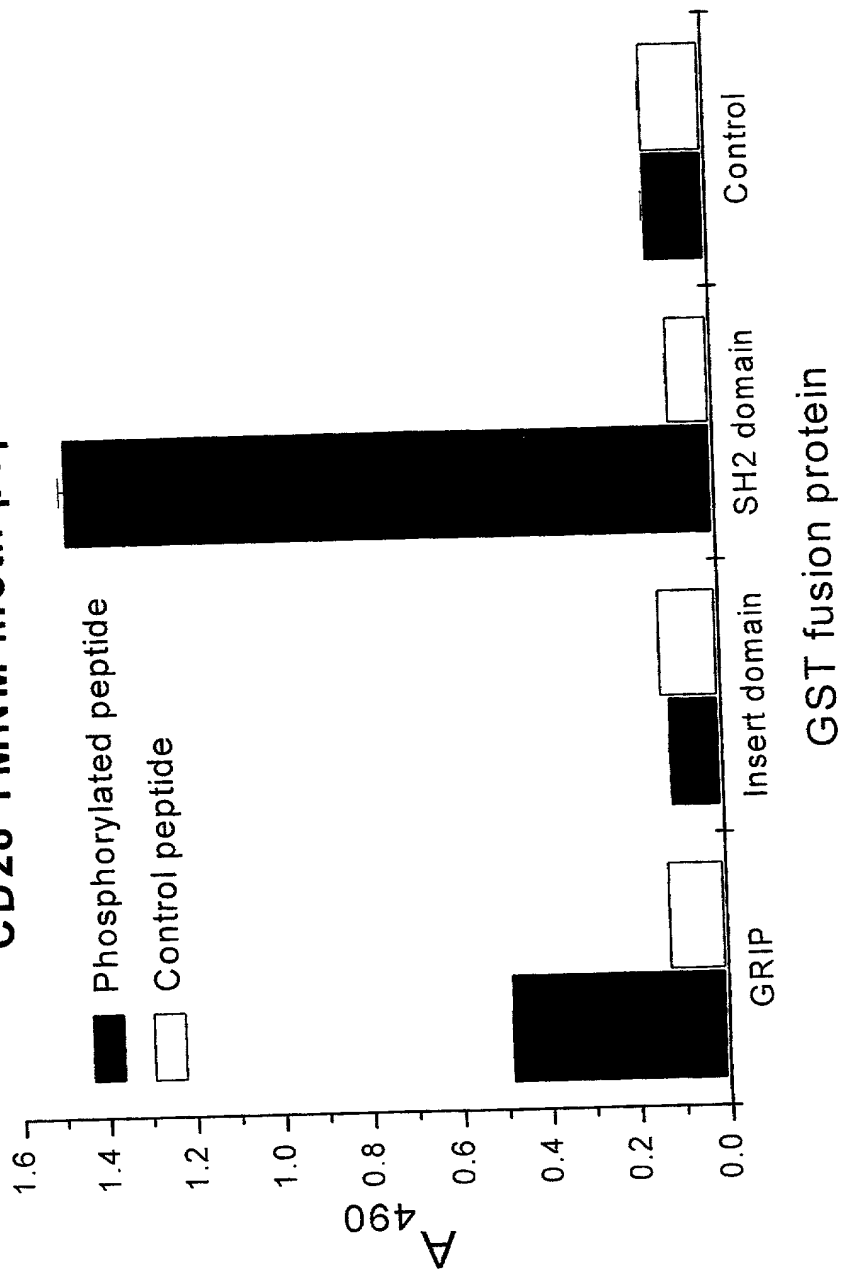
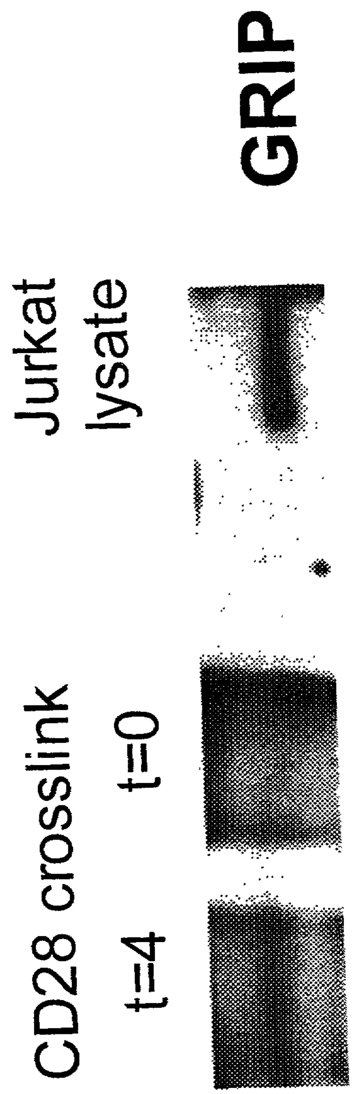


Fig. 13

Association of GRIP with activated CD28 receptor.



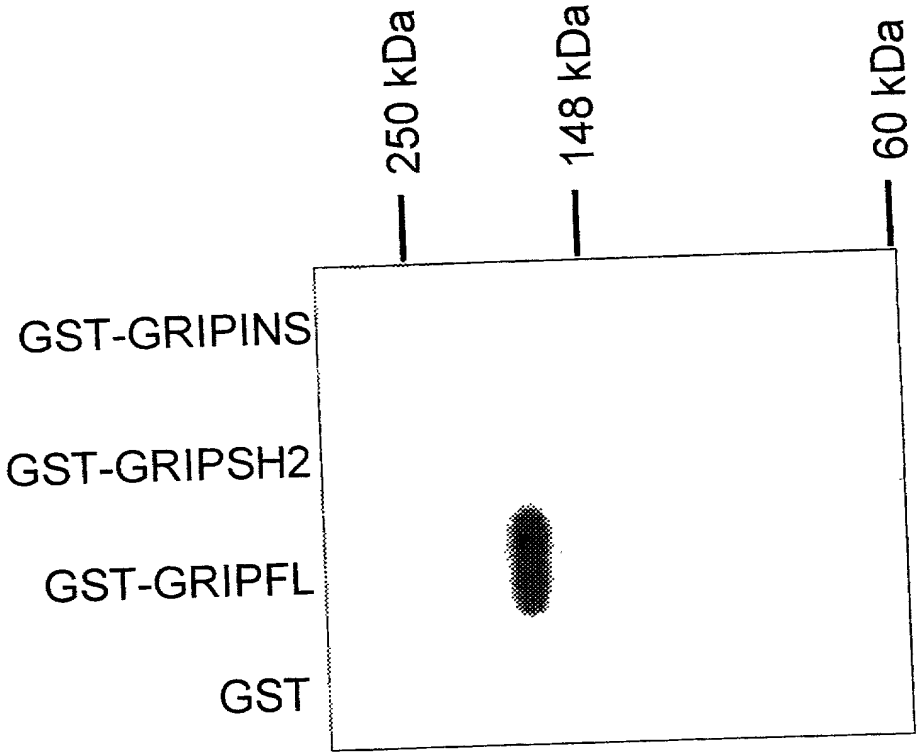


Fig. 14

Association of Sos2 with GRIP

#3

PG3535/USw/0

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER

As below named inventor. I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:
GRIP, HUMAN ADAPTER PROTEIN RELATED TO THE GRB2 FAMILY MEMBER

the specification of which (check only one item below):

[ ]is attached hereto

[ ] was filed as United States application Serial No _____on_____and was amended on _____(if applicable).

[X] was filed as PCT international application Number PCT.GB99.02738 on 18-Aug-1999

and was amended under PCT Article 19 on _____(if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 and all information which became available between the filing of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under Title 35, United States Code. §119 (a)-(d) or §365(b) of any foreign applications(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) having a filing date before that of the application(s) on which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
1. United Kingdom	9818124.1	19-Aug-1998	
2.			
3.			
4.			
5.			

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below:

Application No..	Filing Date (MM/DD/YYYY)	
1.		
2.		
3.		
4.		
5.		

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Continued – Includes References to PCT International Applications)

ATTORNEY'S DOCKET NUMBER

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365(c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S.FILING NUMBERS ASSIGNED (if any)			
PCT.GB99.02738	18-Aug-1999			x	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believe to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334.

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   First **Middle Initial** **Family Name** **Citizenship**
   Residence (City) WARE **(State/Foreign Country)** UK GBX
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   Inventor's Name (typed)
   First **Middle Initial** **Family Name** **Citizenship**
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3. Inventor's signature _____ Date _____
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First **Middle Initial** **Family Name** **Citizenship**

Residence (City) _____ **(State/Foreign Country)** _____
Post Office Address

4. Inventor's signature _____ Date _____
Inventor's Name (typed)
First **Middle Initial** **Family Name** **Citizenship**

Residence (City) _____ **(State/Foreign Country)** _____
Post Office Address

5. Inventor's signature _____ Date _____
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First **Middle Initial** **Family Name** **Citizenship**

Residence (City) _____ **(State/Foreign Country)** _____
Post Office Address

6. Inventor's signature _____ Date _____
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First **Middle Initial** **Family Name** **Citizenship**

Residence (City) _____ **(State/Foreign Country)** _____
Post Office Address

7. Inventor's signature _____ Date _____
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First **Middle Initial** **Family Name** **Citizenship**

Residence (City) _____ **(State/Foreign Country)** _____
Post Office Address

8. Inventor's signature _____ Date _____
Inventor's Name (typed)
First **Middle Initial** **Family Name** **Citizenship**

Residence (City) _____ **(State/Foreign Country)** _____
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SEQUENCE LISTING

<110> Ellis, Jon H.

<120> GRIP, human adapter protein related to the GRB2 family member

<130> 1430-263

<140> US 09/763,024
<141> 2001-02-16

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<222> (5)..(133)

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Ala Arg Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn
1 5 10 15

atg act cca cgt aga ccg ggt cca acg aga aag cat tac cag ccc tat 97
Met Thr Pro Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr
20 25 30

gca cca cct aga gac ttc gca gcc tat cgc tcc tga gcggccgcag 143
Ala Pro Pro Arg Asp Phe Ala Ala Tyr Arg Ser
35 40

cgcgcgatg 152

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<213> Artificial Sequence

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Ala Arg Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn Met
1 5 10 15

Thr Pro Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr Ala
20 25 30

Pro Pro Arg Asp Phe Ala Ala Tyr Arg Ser
35 40

<210> 3
<211> 152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Artificial gene encoding the CD28 cytoplasmic domain

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gctggtaatg ctttctcgtt ggacccggtc tacgtggagt catgttcatg tagtcactgt 120
gcaggagcct gctcctctta ctgcgcgcga tg 152

<210> 4
<211> 993
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(993)

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Met Glu Ala Val Ala Lys Phe Asp Phe Thr Ala Ser Gly Glu Asp Glu
1 5 10 15

ctg agc ttt cac act gga gat gtt ttg aag att tta agt aac caa gag 96
Leu Ser Phe His Thr Gly Asp Val Leu Lys Ile Leu Ser Asn Gln Glu
20 25 30

gag tgg ttt aag gcg gag ctt ggg agc cag gaa gga tat gtg ccc aag 144
Glu Trp Phe Lys Ala Glu Leu Gly Ser Gln Glu Gly Tyr Val Pro Lys
35 40 45

aat ttc ata gac atc cag ttt ccc aaa tgg ttt cac gaa ggc ctc tct 192
Asn Phe Ile Asp Ile Gln Phe Pro Lys Trp Phe His Glu Gly Leu Ser
50 55 60

cga cac cag gca gag aac tta ctc atg ggc aag gag gtt ggc ttc ttc 240
Arg His Gln Ala Glu Asn Leu Leu Met Gly Lys Glu Val Gly Phe Phe
65 70 75 80

```
atc atc cgg gcc agc cag agc tcc cca ggg gac ttc tcc atc tct gtc    288
Ile Ile Arg Ala Ser Gln Ser Ser Pro Gly Asp Phe Ser Ile Ser Val
                 85                  90                  95

agg cat gag gat gac gtt caa cac ttc aag gtc atg cga gac aac aag    336
Arg His Glu Asp Asp Val Gln His Phe Lys Val Met Arg Asp Asn Lys
            100                 105                 110

ggt aat tac ttt ctg tgg act gag aag ttt cct tcc cta aat aag ctg    384
Gly Asn Tyr Phe Leu Trp Thr Glu Lys Phe Pro Ser Leu Asn Lys Leu
        115                 120                 125

gta gac tac tac agg aca aat tcc atc tcc aga cag aag cag atc ttc    432
Val Asp Tyr Tyr Arg Thr Asn Ser Ile Ser Arg Gln Lys Gln Ile Phe
    130                 135                 140

ctt aga gac aga acc cga gaa gac cag ggt cac cgg ggc aac agc ctg    480
Leu Arg Asp Arg Thr Arg Glu Asp Gln Gly His Arg Gly Asn Ser Leu
145                 150                 155                 160

gac cgg agg tcc cag gga ggc cca cac ctc agt ggg gct gtg gga gaa    528
Asp Arg Arg Ser Gln Gly Gly Pro His Leu Ser Gly Ala Val Gly Glu
                165                 170                 175

gaa atc cga cct tcg atg aac cgg aag ctg tcg gat cac ccc ccg acc    576
Glu Ile Arg Pro Ser Met Asn Arg Lys Leu Ser Asp His Pro Pro Thr
            180                 185                 190

ctt ccc ctg cag cag cac cag cac cag cca cag cct ccg caa tat gcc    624
Leu Pro Leu Gln Gln His Gln His Gln Pro Gln Pro Pro Gln Tyr Ala
        195                 200                 205

cca gcg ccc cag cag ctg cag cag ccc cca cag cag cga tat ctg cag    672
Pro Ala Pro Gln Gln Leu Gln Gln Pro Pro Gln Gln Arg Tyr Leu Gln
    210                 215                 220

cac cac cat ttc cac cag gaa cgc cga gga ggc agc ctt gac ata aat    720
His His His Phe His Gln Glu Arg Arg Gly Gly Ser Leu Asp Ile Asn
225                 230                 235                 240

gat ggg cat tgt ggc acc ggc ttg ggc agt gaa atg aat gcg gcc ctc    768
Asp Gly His Cys Gly Thr Gly Leu Gly Ser Glu Met Asn Ala Ala Leu
                245                 250                 255

atg cat cgg aga cac aca gac cca gtg cag ctc cag gcg gca ggg cga    816
Met His Arg Arg His Thr Asp Pro Val Gln Leu Gln Ala Ala Gly Arg
            260                 265                 270

gtg cgg tgg gcc cgg gcg ctg tat gac ttt gag gcc ctg gag gat gac    864
Val Arg Trp Ala Arg Ala Leu Tyr Asp Phe Glu Ala Leu Glu Asp Asp
        275                 280                 285

gag ctg ggg ttc cac agc ggg gag gtg gtg gag gtc ctg gat agc tcc    912
Glu Leu Gly Phe His Ser Gly Glu Val Val Glu Val Leu Asp Ser Ser
    290                 295                 300
```

```
aac cca tcc tgg tgg acc ggc cgc ctg cac aac aag ctg ggc ttc ttc   960
Asn Pro Ser Trp Trp Thr Gly Arg Leu His Asn Lys Leu Gly Phe Phe
305                 310                 315                 320

cct gcc aac tac gtg gca ccc atg acc cga taa                       993
Pro Ala Asn Tyr Val Ala Pro Met Thr Arg
                325                 330
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<210> 5
<211> 330
<212> PRT
<213> Homo sapiens

<400> 5

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Met Glu Ala Val Ala Lys Phe Asp Phe Thr Ala Ser Gly Glu Asp Glu
  1               5                  10                  15

Leu Ser Phe His Thr Gly Asp Val Leu Lys Ile Leu Ser Asn Gln Glu
             20                  25                  30

Glu Trp Phe Lys Ala Glu Leu Gly Ser Gln Glu Gly Tyr Val Pro Lys
         35                  40                  45

Asn Phe Ile Asp Ile Gln Phe Pro Lys Trp Phe His Glu Gly Leu Ser
     50                  55                  60

Arg His Gln Ala Glu Asn Leu Leu Met Gly Lys Glu Val Gly Phe Phe
 65                  70                  75                  80

Ile Ile Arg Ala Ser Gln Ser Ser Pro Gly Asp Phe Ser Ile Ser Val
                 85                  90                  95

Arg His Glu Asp Asp Val Gln His Phe Lys Val Met Arg Asp Asn Lys
            100                 105                 110

Gly Asn Tyr Phe Leu Trp Thr Glu Lys Phe Pro Ser Leu Asn Lys Leu
        115                 120                 125

Val Asp Tyr Tyr Arg Thr Asn Ser Ile Ser Arg Gln Lys Gln Ile Phe
    130                 135                 140

Leu Arg Asp Arg Thr Arg Glu Asp Gln Gly His Arg Gly Asn Ser Leu
145                 150                 155                 160

Asp Arg Arg Ser Gln Gly Gly Pro His Leu Ser Gly Ala Val Gly Glu
                165                 170                 175

Glu Ile Arg Pro Ser Met Asn Arg Lys Leu Ser Asp His Pro Pro Thr
            180                 185                 190

Leu Pro Leu Gln Gln His Gln His Gln Pro Gln Pro Pro Gln Tyr Ala
        195                 200                 205

Pro Ala Pro Gln Gln Leu Gln Gln Pro Pro Gln Gln Arg Tyr Leu Gln
    210                 215                 220
```

```
His His His Phe His Gln Glu Arg Arg Gly Gly Ser Leu Asp Ile Asn
225                 230                 235                 240

Asp Gly His Cys Gly Thr Gly Leu Gly Ser Glu Met Asn Ala Ala Leu
                245                 250                 255

Met His Arg Arg His Thr Asp Pro Val Gln Leu Gln Ala Ala Gly Arg
            260                 265                 270

Val Arg Trp Ala Arg Ala Leu Tyr Asp Phe Glu Ala Leu Glu Asp Asp
        275                 280                 285

Glu Leu Gly Phe His Ser Gly Glu Val Val Glu Val Leu Asp Ser Ser
    290                 295                 300

Asn Pro Ser Trp Trp Thr Gly Arg Leu His Asn Lys Leu Gly Phe Phe
305                 310                 315                 320

Pro Ala Asn Tyr Val Ala Pro Met Thr Arg
                325                 330
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<210> 6
<211> 993
<212> DNA
<213> Homo sapiens

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gccggtccac caggatgggt tggagctatc caggacctcc accacctccc cgctgtggaa 120
ccccagctcg tcatcctcca gggcctcaaa gtcatacagc gcccgggccc accgcactcg 180
ccctgccgcc tggagctgca ctgggtctgt gtgtctccga tgcatgaggg ccgcattcat 240
ttcactgccc aagccggtgc cacaatgccc atcatttatg tcaaggctgc ctcctcggcg 300
ttcctggtgg aaatggtggt gctgcagata tcgctgctgt gggggctgct gcagctgctg 360
gggcgctggg gcatattgcg gaggctgtgg ctggtgctgg tgctgctgca ggggaagggt 420
cgggggggtga tccgacagct tccggttcat cgaaggtcgg atttcttctc ccacagcccc 480
actgaggtgt gggcctccct gggacctccg gtccaggctg ttgccccggt gaccctggtc 540
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gtagtctacc agcttattta gggaaggaaa cttctcagtc cacagaaagt aattaccctt 660
gttgtctcgc atgaccttga agtgttgaac gtcatcctca tgcctgacag agatggagaa 720
gtcccctggg gagctctggc tggcccggat gatgaagaag ccaacctcct tgcccatgag 780
taagttctct gcctggtgtc gagagaggcc ttcgtgaaac catttgggaa actggatgtc 840
tatgaaattc ttgggcacat atccttcctg gctcccaagc tccgccttaa accactcctc 900
ttggttactt aaaatcttca aaacatctcc agtgtgaaag ctcagttcat cctcacctga 960
agcagtgaaa tcaaacttgg caacagcttc cat                                993
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<220>
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<222> (9)..(239)

<220>
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         Met Gly Gly Ser His His His His His His Gly Met Ala Ser
           1               5                  10

atg act ggt gga cag caa atg ggt cgg gat ctg tac gac gat gac gat   98
Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp
 15                  20                  25                  30

aag tct aga gga tcc aag ctt atg gaa gct gtt gcc aag ttt gat ttc   146
Lys Ser Arg Gly Ser Lys Leu Met Glu Ala Val Ala Lys Phe Asp Phe
                 35                  40                  45

act gct tca ggt gag gat gaa ctg agc ttt cac act gga gat gtt ttg   194
Thr Ala Ser Gly Glu Asp Glu Leu Ser Phe His Thr Gly Asp Val Leu
             50                  55                  60

aag att tta agt aac caa gag gag tgg ttt aag gcg gag ctt ggg a     240
Lys Ile Leu Ser Asn Gln Glu Glu Trp Phe Lys Ala Glu Leu Gly
         65                  70                  75
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<210> 8
<211> 77
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic construct

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  1               5                  10                  15

Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Ser
             20                  25                  30

Arg Gly Ser Lys Leu Met Glu Ala Val Ala Lys Phe Asp Phe Thr Ala
         35                  40                  45

Ser Gly Glu Asp Glu Leu Ser Phe His Thr Gly Asp Val Leu Lys Ile
     50                  55                  60

Leu Ser Asn Gln Glu Glu Trp Phe Lys Ala Glu Leu Gly
 65                  70                  75
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<210> 9
<211> 240
<212> DNA
<213> Artificial Sequence

<220>
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construct

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tgtgaaagct cagttcatcc tcacctgaag cagtgaaatc aaacttggca acagcttcca 120
taagcttgga tcctctagac ttatcgtcat cgtcgtacag atcccgaccc atttgctgtc 180
caccagtcat gctagccata ccatgatgat gatgatgatg agaacccccc atggtaagct 240

<210> 10
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
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<222> (1)
<223> lysyl residue bearing a biotin moiety

<220>
<223> Description of Artificial Sequence: Synthesised
peptide

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Lys Leu Leu His Ser Asp Tyr Met Asn Met Thr Pro Arg
1 5 10

<210> 11
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
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<222> (1)
<223> lysyl residue bearing a biotin moiety

<220>
<221> MOD_RES
<222> (7)
<223> PHOSPHORYLATION

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peptide

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Lys Leu Leu His Ser Asp Tyr Met Asn Met Thr
1 5 10

<210> 12
<211> 81
<212> DNA
<213> Artificial Sequence

<220>
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<222> (1)..(81)

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Ala Arg Ser Thr Ala Met Val Ser Met Asn Ser Cys Ser Pro Ala Arg
1 5 10 15

tct gga tct act agt gcg gcc gcc acc gcg gtg 81
Ser Gly Ser Thr Ser Ala Ala Ala Thr Ala Val
20 25

<210> 13
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<212> PRT
<213> Artificial Sequence

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Ala Arg Ser Thr Ala Met Val Ser Met Asn Ser Cys Ser Pro Ala Arg
1 5 10 15

Ser Gly Ser Thr Ser Ala Ala Ala Thr Ala Val
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<210> 14
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His Met Ala Met Glu Ala Pro Ala Arg Ser Gly Ser Val Asp Leu Gln
1 5 10 15

cca agc taa 57
Pro Ser

<210> 15
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Polylinker

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His Met Ala Met Glu Ala Pro Ala Arg Ser Gly Ser Val Asp Leu Gln
1 5 10 15

Pro Ser

<210> 16
<211> 63
<212> DNA
<213> Artificial Sequence

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<222> (1)..(63)

<220>
<223> Description of Artificial Sequence: Polylinker

<400> 16
cat atg gcc atg gag gcc ccg ggg atc gga tcc gat ccg aat tcg agc 48
His Met Ala Met Glu Ala Pro Gly Ile Gly Ser Asp Pro Asn Ser Ser
1 5 10 15

tcg aga gat cta tga 63
Ser Arg Asp Leu
20

<210> 17
<211> 20
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Polylinker

<400> 17
His Met Ala Met Glu Ala Pro Gly Ile Gly Ser Asp Pro Asn Ser Ser
1 5 10 15

Ser Arg Asp Leu
20

<210> 18
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 18
agggaattcc tcgagtcatt ggggagtttc tgcattttct ag 42

<210> 19
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 19
catcgcgcgc agtaagagga gcaggctcct gcacagtg 38

<210> 20
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 20
tctcgttgga cccggtctac gtggagtcat gttcatgtag tcactgtgca ggagcctg 58

<210> 21
<211> 56
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 21
ccgggtccaa cgagaaagca ttaccagccc tatgcaccac ctagagactt cgcagc 56

<210> 22
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 22
catcgcgcgc tgcggccgct caggagcgat aggctgcgaa gtctctagg 49

<210> 23
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 23
tctcgttgga cccggtctac gtggagtcat gttcatgaag tcactgtgca ggagcctg 58

<210> 24
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 24
tctcgttgga cccgctctac gtgcagtcat gttcatgtag tcactgtgca ggagcctg 58

<210> 25
<211> 56
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 25
gcgggtccaa cgagaaagca ttaccaggcc tatgcagcac ctagagactt cgcagc 56

<210> 26
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 26
catcggatcc gaagatttgc cccatcatg 29

<210> 27
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 27
catcgaattc tcatcgcctc tgctgtgc 28

<210> 28
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 28
catcgcgcgc agtaagagga gcaggctcct gcac 34

<210> 29
<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 29
tctcgttgga cccggtctac gtggagtcat gttcacgtag tcactgtgca ggagcctgct 60
cc 62

<210> 30
<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 30
tctcgttgga cccggtctac gtggagtcat tttcatgtag tcactgtgca ggagcctgct 60
cc 62

<210> 31
<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 31
tctcgttgga cccggtctac gtggagtcat tttcacgtag tcactgtgca ggagcctgct 60
cc 62

<210> 32
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 32
catcgcgcgc ggatccaagc ttatggaagc tgttgccaag tttgatttc 49

<210> 33
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 33
catcgaattc gtcgacgcgg ccgcttatcg ggtcatgggt gccacgta 48

<210> 34
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 34
catcggatcc atagacatcc agtttcccaa atgg 34

<210> 35
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 35
catcgaattc ttactggtct tctcgggttc tgtc 34

<210> 36
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 36
catcggatcc ttccttagag acagaacccg agaa 34

<210> 37
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 37
catcgaattc ttaccaccgc actcgccctg ccgcctg 37

<210> 38
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 38
catcgcggcc gcgtcgacga attcttatcg ggtcatgggt gccacgta 48

<210> 39
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 39
accacagtcc atgccatcac 20

<210> 40
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 40
tccaccaccc tgttgctgta 20

<210> 41
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 41
gatctgtacg acgatgacga taagtctaga g 31

<210> 42
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 42
gatcctctag acttatcgtc atcgtcgtac a 31

<210> 43
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 43
gatggaattc agcacacagg acctcaccat ggggggttct catcatc 47

<210> 44
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 44
gatggaattc ttatcgggtc atgggtgc 28

<210> 45
<211> 77
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 45
gatggaattc agcacacagg acctcaccat gtacccatac gatgttccag attacgctga 60
agctgttgcc aagtttg 77

<210> 46
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 46
ataagatggc gcgcggatcc ttacataaac taagtgaaga g 41